

the procedure adopted for sputum may be necessary due to the nature of the material (urine, gastric washings, pus, cerebrospinal fluid, pleural fluid).

2. *Microscopic.*—(a) *Direct.*—Select a small purulent particle of sputum and : : : : a thin film, allow to dry, fix by passi : : : : and stain by the Ziehl-Neelsen meth : : : : pical acid-fast bacilli which will appear red in contrast to the blue of the counterstain of all other bacteria, cells and débris.

(b) *Examination of Sediments.*—Centrifugalize spinal fluid or urine. Then prepare and stain films of the sediment as described above. At times it may be necessary to use a thin film of egg albumin (sterile) on the slide as a fixative to prevent the sediment being washed away during the staining process. Also treat in this fashion the sediments obtained by the concentration methods.

3. *Cultural.*—Cultural methods are being increasingly used routinely in the search for *Myco. tuberculosis*. With proper media rightly handled the results are about as good as obtained by animal inoculation, although cultural methods always leave something to be desired since they do not establish the virulence of the acid-fast organism cultured. Original specimens almost always contain bacteria other than the tubercle bacilli hence it is desirable to treat them, not only to effect concentration, but to eliminate contaminations. The sediments obtained in the concentration methods described above may be used. MacNabb, however, prefers the use of 3 per cent hydrochloric acid. The specimen is placed in a stoppered sterile test tube and an equal volume of 3 per cent hydrochloric acid is added which contains bromcresol purple as an indicator. The contents of the tube are well mixed with a side-to-side motion (to avoid getting the material on the upper tube walls or on the cotton plug) and the specimen is left at room temperature for two hours. Then using 3 per cent sodium hydroxide the specimen is rendered slightly alkaline to the bromcresol purple indicator. Add this reagent aseptically and if at any time there is the slightest question about the aseptic condition of the tube and its plug flame the tube well to within an inch of the upper level of the liquid and insert a fresh sterile cotton plug in the tube. Bromcresol purple changes color at pH 6.8, but it is used in preference to bromthymol blue since it is difficult to determine the pH with bromthymol blue in turbid specimens.

In case the specimen is a clear fluid (pleural fluid, etc.) it is desirable to form a precipitate for the treatment described above which can be done at the outset by the use of 2 cc. of 5 per cent potassium alum. Sometimes transudates show the presence of a coagulum. In such cases the clot may be manipulated to free the fluid from it, or it may be digested as noted above. Laryngeal swabs are given the hydrochloric acid treatment to destroy contaminating bacteria. In the hydrochloric acid treatment the tube is centrifugalized following the neutralization with sodium hydroxide. The sediment remaining is used for the inoculation of culture media.

MacNabb inoculates beef infusion broth from each treated specimen to check on the elimination of all non-acid fast bacteria. If these cultures show growth he retreats the specimen with acid and alkali. If the specimen is satisfactory inoculation is made of 0.25 cc. of the sediment on each tube of the special media used for the growth of the tubercle bacillus. The

substrates he has found most valuable in his work are Dorset's egg medium, and such selective or restraining media as that of Bateman and Fennell or Woolley. He finds Lowenstein's medium suitable for the isolation of tubercle bacilli of bovine origin. The inoculated tubes are covered with a rubber cap in addition to the cotton plug and they are incubated at 37° for eight weeks before reporting a negative result. Other media of excellent reputation are Petroff's and Corper's.

In view of the length of time involved in these routine cultural procedures considerable interest attaches to the rapid culture method recently described by Pryce (1941) who smears the material to be cultured on glass slides which are dried, placed in Petri dishes, and flooded with 15 per cent sulfuric acid for five minutes. The acid is then removed and the preparation carefully washed with sterile distilled water to remove the acid. Next is added the culture medium which may be equal parts of citrated blood and 1 per cent saponin to a total volume sufficient to cover the slide (about 10 cc.). The preparation is then incubated at 37° C. With positive specimens growth of filaments of acid-fast bacteria recognizable with 200 magnifications may be expected within a week. Examination is made by removing the culture fluid, washing the preparation and staining it by the Ziehl-Neelsen method. This method offers promise for early diagnosis and deserves further testing, study and improvement.

The most delicate and valuable method for the diagnosis of tuberculosis although cultural is the following instructions are from MacNabb:

A. Use healthy guinea pigs which have an area of preferably white skin a little over an inch in diameter where a tuberculin test may be done.

B. One week prior to injection of the specimen do an intracutaneous tuberculin test using Old Tuberculin. Inject 0.1 cc. of a freshly prepared dilution so that the guinea pig will receive 1 mg. of Old Tuberculin in this amount.

C. After three days examine for reaction and if negative use the pig for the test.

D. Inject 1.5 cc. of the specimen into the muscles of the left thigh.

E. After four weeks or later perform a second tuberculin test.

F. If the test is positive and in agreement with the cultural test, report as positive.

G. If the tuberculin test is positive and cultural result negative, hold the report for a further four weeks to determine whether or not the culture will be positive at that time.

H. After eight weeks do an autopsy on positively reacting pigs to determine the extent of involvement of the lymphatic glands and spleen, and on negatively reacting pigs to exclude the presence of tuberculous lesions.

Both culture and animal inoculations should be done on those specimens where a second specimen may be difficult to obtain, as ureter samples, pus, spinal fluid, ascitic fluid, etc.

5. *Serological.*—*The Intradermal Tuberculin Test.*—One of the most useful and most neglected tests assisting in the diagnosis of tuberculosis is the Tuberculin Test. At one time it was taught that practically all persons

past the age of puberty were tuberculin positive. Therefore tuberculin tests were only of value in pediatric practice. We now know that large numbers of young adults not suffering from tuberculosis are tuberculin negative. The figure will vary in different parts of the United States but on average 50 per cent of persons under thirty years of age are tuberculin negative and even among older persons a very considerable per cent are likewise tuberculin negative. It is therefore desirable to do a tuberculin test on all persons thought to have tuberculosis. A positive test permits a diagnosis of tuberculosis (but does not make it). There is every reason to question the diagnosis of tuberculosis in a patient properly tested with tuberculin and found negative who is not at the time receiving tuberculin therapy or suffering from intercurrent disease involving skin reactivity (chiefly rash-producing diseases). The test is best carried out using Purified Protein Derivative. The first dose is 0.00002 mg. of tuberculin contained in a volume of 0.1 cc. If after three days the reaction is negative the second dose of 0.005 mg. is administered, also read after three days. A positive reaction is an area of induration greater than 0.5 cm. in diameter read not sooner than forty-eight hours after the intradermal injection and better on the third day. No reaction should be considered positive on the basis of erythema alone; it must be palpable. If Purified Protein Derivative cannot be obtained Old Tuberculin may be used, beginning with a dose of 0.1 cc. of a 1 to 10,000 dilution which may be stepped up ten-fold, usually not more than twice in case of a negative reaction.

Mycobacterium Tuberculosis var. Bovis

The common name is tubercle bacillus, bovine type. The rods are shorter and more plump than the human type, ranging in size from 1 to 1.5 μ . Very short forms are frequently intermixed with somewhat larger forms. Stain irregularly. Acid-fast. Gram-positive. Are less easily cultivated than the human variety. The addition of glycerol to the culture medium does not enhance the growth of the bovine type. It is the cause of tuberculosis in cattle. It is transmissible to man and domestic animals. More highly pathogenic for animals than the human type. If 10 mg. amounts of moist bacilli are injected subcutaneously into rabbits the bovine type causes a generalized fatal infection while the human type produces only a localized infection or none at all.

Other Species of Mycobacterium

The other members of this genus include 11 species. Of this group *Myco. paratuberculosis* causes Johne's disease, a chronic diarrhea in bovines not transmissible to man; *Myco. avium* causes tuberculosis in chickens and is transmissible to pigeons, birds, mice and rabbits but only rarely to man. Several members of the group cause disease in such cold-blooded animals as snakes, turtles, fish and frogs. Other species occur in dairy products while others have been isolated from plant dust and soil. A form, previously known as *Myco. smegmatis*, now held to be identical with *Myco. lacticola*, occurs on the genitalia of man. It is obvious then that harmless acid-fast bacilli occur rather widely in man's environment and that every care must be taken to ensure that a diagnosis of "tubercle bacilli present" has fully considered that fact.

TABLE 71.—DIFFERENTIATION OF THE VARIOUS TYPES OF ACID-FAST ORGANISMS

	<i>Mycobacterium tuberculosis</i>				
	Human	Bovine	Avian	Cold-blooded	Saprophytes
Nutrient agar	37° C. No growth 4 weeks	37° C. No growth 4 weeks	37° C. Scanty growth 4 weeks	20° C. Moderate growth in 7 days	20° or 37° C. Abundant, pig- mented growth in 7 days
Glycerol potato agar	Growth luxur- iant, nodular cream col- ored 4 weeks	Growth scanty, thin, grayish 4 weeks	Growth profuse, thin, creamy 4 weeks	Growth profuse, nodular, cream colored 7 days	Growth abundant pigmented, ap- pearing as dry bread crumbs in 7 days
Grows at.	41° C.	41° C.	43° C.	20° C.	20° or 37° C.
Susceptibility of:					
Guinea pigs	++	++	+-	+	-
Calves	+	++	++	-	-
Rabbits	+	++	+	-	-
Fowls	-	-	++	-	-
Frogs	-	-	-	++	-

Mycobacterium Leprae

The bacillus of leprosy was discovered in 1874 by Hansen (Neisser, 1879) in the leprous tubercles of persons afflicted with leprosy. The bacilli are small slender rods resembling *M. tuberculosis* in form, measuring 0.2 to 0.35 μ in width by 1.5 to 4.6 μ in length. They are straight, rarely bent or curved, and have pointed ends. Gram-positive and acid-fast, in stained preparations they show unstained spaces similar to those of the tubercle bacillus. Although several acid-fast organisms have been isolated from leprous lesions, none of these, with the possible exception of the bacillus of McKinley and Soule, has been definitely proved to be the cause of leprosy.

Examination of Materials.—*Lepra* bacilli may be demonstrated by microscopic examinations of the various lesions of leprosy. Neither cultural examination nor animal inoculation are of any aid in the diagnosis.

Nasal Lesions.—The initial lesion of leprosy is often an ulcer of the mucous membrane, located at the junction of the bony and cartilaginous septum. Collect swabs or scrapings from this or other nasal lesions and prepare smears. If desirable, the patient may be given 60 grains of potassium iodide beforehand to produce a drug coryza and increase the nasal secretions.

Skin Lesions.—With a sterile safety razor blade, held with the corner of the cutting edge over the lesion, quickly make a small incision through the thickened area and without removing the razor, change the direction of the razor by twisting at right angles to the incision; scrape the incision with the corner of the razor blade. From this drop of material on the corner of the razor blade prepare films. Material may also be obtained by means of a capillary pipet, a syringe, or by scraping with a scalpel.

1. Microscopic.—Fix the smears obtained and stain by the Ziehl-Neelsen method for acid-fast bacilli. Care must be taken not to carry the decolorization too far because the *lepra* bacilli are more easily decolorized than *M. tuberculosis*. The typical package bundles of *lepra* bacilli or organisms packed in *lepra* cells are often found in the nasal smears. In specimens from skin nodules the *lepra* bacilli are found chiefly in the derma, packed in the characteristic *lepra* cells (called foam cells) and engulfed in the endothelial cells lining the lymphatics. *Lepra* bacilli are rarely demonstrable in the anesthetic area of nerve leprosy.

CORYNEBACTERIUM

The genus *Corynebacterium*, to which the diphtheria bacillus belongs, as at present constituted, comprises 21 species described as follows: "Slender, often slightly curved, rods with a tendency to club and pointed forms, with branching forms in old cultures. Barred, uneven staining. Not acid-fast. Gram-positive. Non-motile. Usually aerobic. No endo-

toxin. Produces a powerful exotoxin. Character-

istics, *C. diph-*

theriae, a patho-

logical agent

of the group denomi-

nated as diphtheroids.

This group

is of great importance

and as a rule

has very little pathogenicity due doubtless to the inability of these organisms to elaborate toxin. *C. pyogenes* causes abscesses in cattle, swine and other domestic animals. *C. enzymicum* is pathogenic for rabbits, guinea pigs and mice and has been recovered from the lungs, blood and joints of man. *C. xerose* has been isolated from normal and diseased conjunctiva. *C. acnes*, the acne bacillus, resides in acne pustules. Thus while diphtheroids may occasionally be found in minor inflammatory conditions their real significance is their presence in the environment rendering them frequent contaminants of bacterial cultures and making more difficult the recognition of the true diphtheria bacillus, which some of them closely simulate.

Corynebacterium Diphtheriae

irregular diameter. Edge may be entire or
Gela
Loef
to cre
cular, moist, grayish
with an entire edge.

for months

Metabolism.—Growth at temperatures between 19° and 42° C., with optimum production of toxin at 34° to 35° C., most favorable temperature for vegetative development at 37.5° C. Marked preference for oxygen and free access to air is essential for the cultivation of most strains. Will grow on simple meat extract mediums but does much better on Loeffler's medium (beef blood serum mixed with glucose bouillon in the ratio of 3 to 1, coagulated by heat) or on heated blood agar.

Biochemical Reactions.—No proteolytic activity. All strains produce acid, but no gas, in dextrose and lactose, and generally ferment salicic acid, maltose, dextrin and glycerol. Indol is not for

Pathogenicity.—*Corynebacterium diphtheriæ* produces a powerful soluble toxin. The cause of diphtheria in man and through its toxin, it is pathogenic for the usual laboratory and domesticated animals, and fowls. Rats and mice are resistant unless the toxin is given in large doses. At times the diphtheria bacillus may be the cause of conjunctivitis, wound infection, middle-ear infection, and bronchopneumonia. (See Table 72.)

an avirulent variant in color than the
no the granules are

coarser, with irregular forms of tetrads as dissociation, colony appearance

picture of the disease have stimulated study of the diphtheria bacillus resulting in the recognition of three types, *gravis*, *mitis* and *intermedius*. In Table 72 (from J. W. McLeod, Bact. Rev.) are shown data regarding these three types.

Examination of Clinical Materials.—From suspected clinical cases of diphtheria, collect a portion of the pseudomembrane with forceps or by means of a cotton swab. If membrane formation is absent, or in the case of examination for carriers, swab over the mucous membranes of the pharynx, tonsils, nasal cavities and auditory canals. In the collection of the specimen, avoid contamination if possible by not allowing the swab to touch the tongue or any part of the oral cavity, or to come in contact with excessive nasal or pharyngeal secretions.

1. **Microscopic.**—An immediate presumptive diagnosis can sometimes be made. Smears are prepared from the collected exudate, stained with the appropriate stains (Loeffler's methylene blue or Neisser's stain) and examined for the characteristic described morphology. Since direct smear preparations may be negative for *C. diphtheriæ*, or show the presence of Vincent's organisms, diphtheroids, and other contaminating bacteria, the diagnosis should be confirmed by cultural examination and virulence tests.

2. **Cultural.**—(a) *Rapid Method of Brahdý.*—Brahdý and associates described (1934) a modified Folger-Solé culture method for the diagnosis of diphtheria in which in 95 per cent of the cases accurate reports can be rendered in four hours with a considerable number (80 per cent) possible in two hours. Sterile cotton swabs are impregnated thoroughly with undiluted, unheated, horse serum to which no preservative has been added. The swabs are then squeezed lightly against the sides of the tube to remove any surplus serum. They are removed and rotated by the fingers in a flame to secure at least surface coagulation of the serum and possibly to destroy any serum antibodies. These swabs are then utilized to take the ordinary routine nose and throat cultures of the suspected case. They are then placed in dry sterile tubes in the incubator and examined at the end of two and four hours by smear preparations. At the end of four hours Loeffler's slants may be inoculated from these swabs for isolation, control and subsequent identification.

TABLE 72.—MOST RELIABLE CRITERIA IN DIFFERENTIATING THE THREE TYPES OF *CORYNEBACTERIUM DIPHTHERIE* (McLeod)

	MITIS			INTERMEDIUS		GRAVIS	
	<i>Long forms; metachromatic granules</i>			<i>Barred forms often long and clubbed at ends</i>		<i>Short forms tending to stain uniformly and sometimes closely resembling Hofmann's bacillus</i>	
1. Morphology							
2. Appearance of growth on heated blood agar	Fairly abundant moist, relatively smooth, semi-opaque and glistening colonies			<i>Flat, fine, dry, opaque, and associated with delicate olive green discoloration of medium</i>		Abundant, flat, dry, matted, relatively opaque	
3. Appearance of growth on special blood tellurite media	Smooth, convex, medium-sized, with black centre and semi-transparent gray periphery for first 50 hours; finer and larger colonies			<i>Flat, fine, dull with black center and often small central papilla; gray periphery with slightly raised margin; colonies very uniform in size</i>		Medium to large with slight to marked radial striations and slightly to markedly indented periphery; color varying from gray-black to black; finer and larger colonies	
4. Consistence of colonies	Approximately that of warm butter. Colony smears under needle and forms homogeneous suspensions			Intermediate between <i>gravis</i> and <i>mitis</i>		Approximate to that of cold margarine, colony is pushed in front of needle and tends to fracture	
5. Appearance of growth in nutrient broth	Heavy uniform or mixed uniform and granular turbidity; pellicle late, soft and leaving ring on side of tube			<i>Finely granular turbidity, settling to loose clear supernatant</i>		All variations from clear fluid with marked pellicle broken by agitation to coarse flakes which settle to base of tube to slight pellicle over abundant fine turbidity mixed with granules and flakes of varying size	
6. Hemolytic activity on blood agar plates	Distinct			<i>Absent</i>		Variable	
7. Fermentation of starch and glycogen	Negative			<i>Negative</i>		Positive	
8. Regularity of pathogenic action in guinea pigs	10%-20% of non-pathogenic strains (high pathogenicity for mice)			10% non-pathogenic (low pathogenicity for mice and for spermophils)		Non-pathogenic strains extremely rare (moderate pathogenicity for mice)	
9. Antigenic homogeneity or diversity	Great diversity of antigenic groups			Antigenically homogeneous		Two main antigenic groups each of which has been found as an epidemic strain over wide areas	

(b) *Conventional Method*.—The swab obtained from the patient is used for the immediate inoculation of a slant of Loeffler's serum which is incubated at 37° C. for eighteen to twenty-four hours, or shipped at once to a distant laboratory for study. Following inoculation of the Loeffler's slant the swab may be spread (by rolling) across the surface of a slide for direct stain examination.

It may also be desirable to inoculate a blood agar plate for incubation at 37° C. for twenty-four hours. This will provide information on the general bacterial flora of the area swabbed, particularly streptococci, and will give opportunity for notation of colony form and for single colony isolation of diphtheria-like bacilli. The inoculation of a plate of McLeod's chocolate-tellurite agar or of Fraser's cystine-tellurite-unheated blood agar will provide a means for the study of the *gravis*, *mitis* and *intermedius* types. (See Table 72.)

If typical diphtheria bacilli are found, and the culture is from a suspected case, a presumptive diagnosis should be made at once. In the examination of cultures from suspected carriers, diphtheria-like bacilli should be further identified by fermentation and virulence tests. The main differences between species important for man as revealed in culture are tabulated below:

	Dextrose	Sucrose	Dextrin	Hemolysis	Virulence
<i>C. diphtheriæ</i>	+	—	+	Beta	+
<i>C. pseudodiphthericum</i>	—	—	—	—	—
<i>C. xerose</i>	+	+	—	—	—

It should be remembered, however, that there are avirulent strains of *C. diphtheriæ* and that there are diphtheroids other than Hoffmann's bacillus and *C. xerose* which ferment dextrose but fail to ferment sucrose.

3. *Animal Inoculation (Confirmatory Virulence Test)*.—This is the only certain method by which the identity and virulence of *C. diphtheriæ* can be confirmed, or by which it can be distinguished from non-virulent variants.

(a) *Guinea Pig Test*.—*C. diphtheriæ* is the only known species of the genus *Corynebacterium* which produces a fatal toxemia in guinea pigs. Either the subcutaneous or intracutaneous test may be employed.

(1) *Subcutaneous*.—Inject 2 cc. of a pure culture grown for forty-eight hours in infusion broth, or 4 cc. of a suspension of a Loeffler's slant growth in 10 cc. of saline, subcutaneously into a 250-gm. guinea pig. At the same time a similar injection of the culture is made into a control guinea pig which is given 250 units of diphtheria antitoxin intraperitoneally twenty-four hours previously. If the organism is a virulent diphtheria bacillus, the unprotected animal will die within three to five days, and on post-mortem, show local edema and enlarged hemorrhagic adrenals, whereas

the control animal has been injected with the same amount of culture twenty-four hours previously. The growth from a twenty-four hour Loeffler's slant is suspended in 20 cc. of normal saline, and 0.15 cc. are injected into the shaved abdominal skin of each guinea pig. Virulent strains of diphtheria bacilli produce a local circumscribed infiltrated lesion which shows a characteristic superficial necrosis in two or three days. The site of inoculation on the protected animal appears normal. Several tests may be carried out using one pair of guinea pigs by this method.

(b) *Frobisher's Chick Test*.—Frobisher (1910) showed that subcutaneous

avirulent strains or with *C. xerosis* or *C. pseudodiphthericum* they were completely resistant. Diphtheria virulence has customarily been demonstrated with guinea pigs (and rabbits) in which the age factor is less important and the general all around laboratory usefulness greater than in the case of the chick. On the other hand rodents may not always be available and this alternative procedure is to be recommended. The method is to inject 0.5 to 1.0 cc. of a forty-eight hour broth culture into the subcutaneous areolar tissues over the insertion of the right wing of one chick. Another chick prepared by the injection of antitoxin (50 to 100 units) about one hour before the culture is to be tested is necessary to properly control the test. The chicks unprotected by antitoxin which receive virulent cultures of *C. diphtheria* will sicken and die in one, two or three days with few exceptions (2.1 per cent in Frobisher's series) and these will die in a few days more. The few which live five to eight days will show definite wing paralysis by the third day so that positive results are reportable within the usual three-day period.

4. *Susceptibility Test—The Schick Reaction*.—This test gives an indication of the presence or absence of immunity to *C. diphtheria*. Diphtheria toxin is diluted with normal saline previous to use so that one dose (1/50 m.l d) is contained in 0.1 cc. Part of this diluted toxin is heated to 75° C. for five minutes, to serve as a control.

Technic of Test.—Sterilize the flexor surface of one arm and inject 0.1 cc. of the unheated toxin into the superficial layers of the skin (intra-dermal), resulting in a definite wheal. A control injection of 0.1 cc. of heated toxin is made on the flexor surface of the opposite arm. Observations are then made daily for several days, the readings on the fourth day giving the best differentiation between the various reactions.

Types of Reaction.—(a) *Negative Reaction*.—No redness, or infiltration in either arm.

(b) *Positive Reaction*.—No reaction in the control arm. In the test arm a circumscribed slightly raised area of redness, 1 to 2 cm. in diameter, appears in twenty-four to thirty-six hours, reaching its maximum development on the third or fourth day. This lasts for seven to fifteen days, gradually fading to superficial scaling and persistent brownish pigmentation.

(c) *Negative Pseudoreaction*.—An erythematous reaction of the allergic type, developing equally in both arms. It appears earlier, six to eighteen hours, is less sharply circumscribed and less intense than the positive reaction, and usually disappears by the fourth day.

(d) *Positive Combined Reaction*.—The reaction on both arms has the appearance of a pseudoreaction during the first twenty-four hours. After twenty-four to thirty-six hours the reaction in the test arm continues to develop, while the control commences to fade. By the third or fourth day the difference between the test injection and the control is usually quite distinctive.

Positive and positive combined reactions indicate susceptibility to diphtheria, while negative and pseudoreactions indicate sufficient immunity

to protect from all ordinary risks of infection. Adults showing the positive combined reaction must be given special attention when immunized with diphtheria toxoid. In general toxoid should not be given persons over eight years of age without preliminary test to see what reactions if any may be encountered but such precautions will be found particularly valuable in dealing with the individual who has shown a positive combined reaction. Such preliminary test consists of the intradermal injection of a fraction of the proposed dose with observation of results before the full dose is administered.

THE SPIROCHETES

The term "spirochete" has been loosely applied to a large group of flexible, motile, non-flagellated, filamentous, spiral organisms, many of which are saprophytes or commensals and some of which cause serious infections in man and in other animals. They are the causative agents of syphilis, yaws, relapsing fever, and other diseases. They are classified under the order: *Spirochætales*. The organisms of this order are defined as follows: "Protozoön-like in certain characters. Cells usually slender, flexuous spirals; multiplication of cells by transverse division; no conclusive evidence of longitudinal division. Motility often characteristic but without polarity." There is a single family, namely *Spirochætaceæ*, organisms of which have the same characters as those of the order. There are 6 genera: namely, *Spirochæta*, *Saprospira*, *Cristispira*, *Borrelia*, *Treponema* and *Leptospira* which are differentiated as indicated in the following Key.

Key to the Genera of Family Spirochætaceæ

- A. Cells 45 to 500 μ long forming a helix with large, irregular or inconstant coil. Altered but not disintegrated by 10 per cent bile salts.
 1. No crista or ridge.
 - (a) Protoplast wound spirally around a well defined axis filament. No obvious periplast membrane and no loculation. Fresh water and marine forms especially in the presence of H_2S . Genus I. *Spirochæta*.
 - (b) No evident axis filament. Distinct periplast membrane. Found in Foraminifera ooze. Genus II. *Saprospira*.
 2. Crista or ridge present. Periplast membrane demonstrable. Axis filament and loculation seen when stained. Parasite in Mollusks. Genus III. *Cristispira*.
- B. Cells 4 to 15 μ (rarely up to 40 μ in *Leptospira*). Commonly disintegrated by 10 per cent bile salts.
 1. Open irregular coils; stain readily with aniline dyes other than the Giemsa stain. Parasitic, many pathogenic, some transmitted by arthropods. Active lashing movements and slow rotation. Genus IV. *Borrelia*.
 2. Close, permanent coils; difficult to stain with aniline dyes other than the Giemsa stain.
 - (a) Pitch of coils about 1 μ or slightly more. Movements show bending and rotation. Disintegrated by 10 per cent saponin. Parasitic and many pathogenic to mammals. Genus V. *Treponema*.

- (b) Pitch of coils about 0.5μ or slightly less. Movements rapid, spinning and intermittently active lashing. One or both ends recurved. Resistant to 10 per cent saponin. Some parasitic and pathogenic to mammals, others found in water. Genus VI. *Leptospira*.

BORRELIA

The genera *Spirochæta*, *Saprosira* and *Cristispira* are made up of large saprophytic forms which have no medical significance. It may be mentioned, in passing, that the genus name *Spirochæta* was used by Ehrenberg, in 1838, in connection with the species *Spirochæta plicatilis* and hence was never free for use in naming the altogether different form causing syphilis and incorrectly named *Spirochæte pallidum* or *Spirochæta pallida* in 1905. The genus *Borrelia* includes 15 species described as: "Small, parasitic, spiral forms; flexible, with terminal filaments. The spirals are large, wavy, three to five in number." Six species cause relapsing fever: *Borrelia recurrentis*, *B. duttonii*, *B. kochii*, *B. noryi*, *B. berbera*, and *B. carteri*. *B. vincentii* is associated with Vincent's angina and other fusospirochetal infections. *B. refringens*, a harmless form, is found in the blood of man and may be mistaken for *B. gallinarum* causes septicemia. The genus are responsible for minor diseases in lower animals.

Relapsing Fever

Various closely related and clinically identical relapsing fevers, characterized by an initial febrile period of three to ten days followed at intervals of one to fourteen or more days by successive relapses of shorter duration, occur in many regions of the world as shown in Figure 40. The causative organisms of these fevers are morphologically similar, and their differentiation is based largely on immunological differences, and probably on differences in pathogenicity for lower animals. Six species are described in Bergey's Manual. These include *B. recurrentis*, the agent of European relapsing fever, which was described by Obermeier in 1873; also *B. duttonii* (Central Africa), *B. kochii* (East Africa), *B. berbera* (North Africa), *B. carteri* (India) and *B. noryi* (America).

Borrelia Recurrentis

Habitat—The blood and tissues of man.

Motility. Can rotate due to stretching of axial filament by pressure of contracting protoplasm. May rotate rapidly in either direction and move in either direction. Spirals are regular and . . .

paraffin oil seal. Also grown on semi-solid serum agar by Kligler and Robertson (1922).

Resistance.—Ten per cent bile salts cause disintegration, 10 per cent saponin immobilizes organisms in thirty minutes, and breaks them up in a few hours, sometimes leaving axial filaments bare. Resistance to heat and cold variable, remain alive some time at 0° C, killed quickly at 60° C.

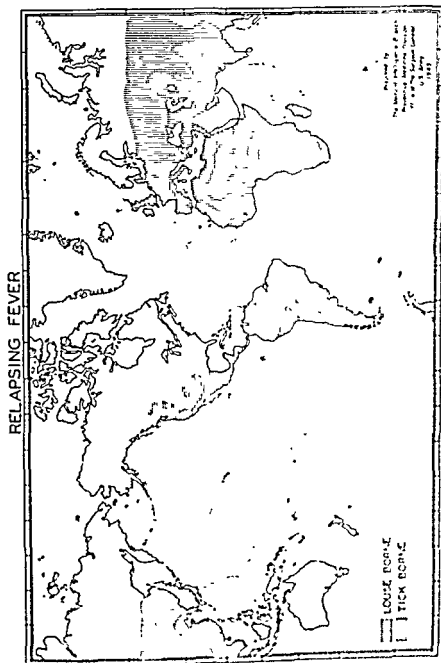


FIG. 10.—World distribution of relapsing fever.

Pathogenicity.—Causes relapsing fever in man. Does, rats and guinea pigs are not susceptible. Transmissible by inoculation of infected blood in man and laboratory animals, including monkeys, rats and mice. In these species bacteria appear in the blood after incubation periods of two to five days. Normal *B. burg* is only mildly pathogenic for monkeys and is non-pathogenic for rats. *B. duttoni* is fatal for monkeys and occasionally for rats. *B. recurrentis* is pathogenic for both, but not fatally so, producing relapses in monkeys and a prolonged fever in rats.

Fusobacterium plauti-vincenti, also known as *Bacillus fusiformis*, which is associated with *Borrelia vincentii* in Vincent's angina and related infections, is classified in Bergey's Manual (1939) under the order: *Eubacteriales*, family: *Bacteriaceae*, genus: *Fusobacterium*.

This genus is defined as follows: "Gram-negative, anaerobic rods usually with tapering ends. Usually motile; stain with more or less distinct granules." According to Zinsser and Bayne-Jones (1939), Smith classified the fusiform bacilli in three types according to morphology, as follows:

"Type I.—This is the large fusiform bacillus found in Vincent's angina. Length 3 to 30 μ , width 0.6 to 0.8 μ . Ends tapered and pointed. Actively motile in materials from lesions and in some young cultures. Produces a rancid odor in cultures. It is suggested by Smith that this fusiform bacillus is identical with the so-called *Spirochæta buccalis*. Has a double contour in darkfield illumination. It is susceptible to arsenic."

"Type II.—Very similar to Type I, thinner (0.3 μ wide), appearing in a single line in darkfield illumination. Actively motile when taken from lesions. Susceptible to arsenic. Smith suggests that in its spiral form it may be identical with Vincent's spirochete."

"Type III.—Small straight form 2 to 5 μ long, 0.3 to 0.4 μ wide. Non-motile. Common in lung abscesses and chronic bronchiectasis."

Examination of Clinical Materials.—Collect fresh material from the ulcerative or other lesions with a sterile platinum loop and make preparations for microscopic examination.

1. **Microscopic.**—Examine fresh material by darkfield illumination, and fixed films, stained with Loeffler's methylene blue, carbolfuchsin, Wright's or Giemsa stain, for the typical spirochetes and fusiform bacilli. The spirochetes are usually slightly longer than the bacilli, and the undulations are shallow, irregular and variable in number. They stain uniformly but less intensely than the bacilli which characteristically show deeper color at the ends, and barring or banding in the centers.

2. **Cultures.**—As noted above the organisms can be grown in culture under anaerobic conditions but as a rule the routine diagnosis is based on microscopic examinations alone.

3. **Animal Inoculation.**—Not used as a routine diagnostic procedure.

Related *Borrelia* of Birds and Lower Animals

Species of *Borrelia* morphologically similar to those responsible for the relapsing fevers of man have been observed in a wide variety of diseases of birds and lower animals, in different parts of the world. It seems probable that at least some of these may be identical with the species affecting man and that certain of the lower animal hosts may afford a natural reservoir for the agents of the human disease.

A representative disease of fowls is the spirochetosis of chickens caused by *B. gallinarum* which occurs chiefly in South America. This disease is normally transmitted by ticks, *Argas persicus*, and other species, which transmit the infection through the egg to their young. A similar infection of geese which has been observed especially in Russia and northern Africa is due to *B. anserina*.

These organisms may be differentiated from the spirochetes of human relapsing fever by differences in pathogenicity. *B. anserina* may occasion-

ally infect mammals, but is more pathogenic for birds, and the mammalian forms do not infect birds. It has also been suggested that the avian forms constitute a separate group based on cross-immunity tests. Normally recovered birds are immune as long as the serum contains antibodies. Species affecting cattle, horses and other animals, including *B. theileri* are also morphologically like *B. recurrentis*, and are transmitted by ticks.

The wide distribution of these organisms in nature makes it important to carefully evaluate the results of animal inoculations made for the identification of the spirochetes present in human cases of relapsing fever.

TREPONEMA

The *Treponema* are characterized as: "Parasitic and frequently pathogenic forms with undulating or rigid spirilliform body. Without crista or columella. With or without flagelliform tapering ends." According to Bergey's Manual the genus *Treponema* is made up of 8 species: 6 of these are of little or no medical significance, but may be confused with the two important species, namely *Tr. pallidum*, the cause of syphilis, and *Tr. pertenue*, the etiological agent of yaws.

Treponema Pallidum

Habitat.—The infected tissues and blood of individuals infected with syphilis.

v 6 to 14 μ

nts. Body

about 1 μ

ial filament

not demonstrated and protoplasm appears homogeneous. No crista or undulating membrane. Motile but does not progress far, chiefly rotational with undulations. Division transverse. Not filterable.

Staining.—Can be demonstrated in smears with special stains of Romanowsky type such as Giemsa and Wright's stains; the Levaditi silver impregnation method is useful for demonstration in tissues. Darkfield illumination is used for routine demonstration of treponema in lesions.

Resistance.—Spirochetes from syphilitic lesions rapidly immobilized and later fragmented by distilled water, or 10 per cent saponin. Killed by drying one hour, by heat at 39° C. in five hours, 40° C. in three hours, 41° C. in two hours, or 41.5° C. in one hour. Completely disintegrated by 10 per cent bile salts. Resists trypsin digestion many days.

Culture.—Difficult and not used routinely in the diagnosis of syphilis. The organisms may be grown under anaerobic conditions in Noguchi's ascitic fluid agar or liquid medium in a deep tube containing fresh tissue, but it appears that

Pathogenicity.—Syphilis occurs naturally only in man. Infection can be produced experimentally in apes, monkeys, rabbits and guinea pigs. Rabbits are most commonly used in the laboratory.

Examination of Clinical Materials.—The laboratory examinations commonly used as aids in the diagnosis of syphilis are: microscopic demonstrations, serological tests, and special procedures such as the Wassermann test.

Treponema Pertenu

Tr. pertenue, the spirochete of yaws, is so similar morphologically to *Tr. pallidum* that it cannot be differentiated by the usual microscopic or serological examinations. The spirochetes are demonstrable in material from the cutaneous papules and ulcerations; the Wassermann test is usually positive and the disease is cured more rapidly than is syphilis by arsphenamine.

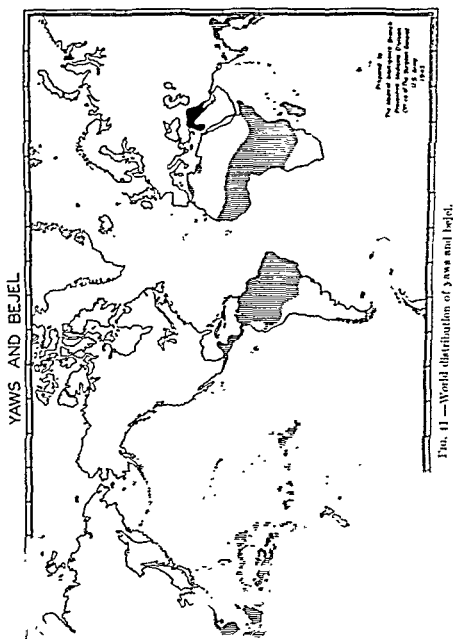


FIG. 41—World distribution of yaws and bejel.

"Bejel" is another "treponematosi" w interior Syria. It has been studied by P and clinically, syphilis, yaws and bejel .

is however much reason to see in them all a rather common biological etiology which is conditioned in the different diseases by the habits of life and geographical environment of the persons concerned. Light may be thrown on this debatable point by the present war which is affording an increasing number of competent observers opportunity to study two, or even all three, of these diseases first hand.

Treponema Carateum

A species of *Treponema*, morphologically identical with *Tr. pertenue* and *Tr. pallidum* is the etiological agent of Pinta, a disease whose manifestations are dermatrophic and which occurs in parts of Central and Northern South America. Known as *Tr. carateum* this species is of interest because of the presence in Pinta of a positive Wassermann.

LEPTOSPIRA

Organisms of the genus *Leptospira* are defined as. "Parasitic forms. Slightly twisted cylinders with flagelliform tapering ends, one extremity being sharply curved into a hook." There are 4 species, viz, *L. biflexa*, an organism isolated from pond water, *L. interrogans*, which was for a time thought to be the cause of yellow fever, *L. hebdomadis*, etiological agent of Japanese seven-day fever; and *L. icterohæmorrhagiae*, which incites infectious jaundice.

Infectious jaundice is widely distributed with another form of whose mode of transmission is not yet certainly known. Weil's disease is most likely to occur in damp, rat-infested places such as mines, old warehouses and docks and trenches as used in World War I. Many outbreaks have been associated with war and the disease has a certain occupational aspect. The leptospira concerned is transmitted from the rat to man through environmental contamination by infective rat urine.

Leptospira Icterohæmorrhagiae

Habitat.—The organs, including the blood and kidneys, of infected wild rats, the blood, liver, adrenal glands, kidneys and urine of patients with infectious jaundice.

Morphology.—The spirochetes are cylindrical in shape, 0.25 to 0.3 μ in width by 6 to 9 μ , and exceptionally 20 to 25 μ in length, with pointed ends. Spiral amplitude 0.4 to 0.5 μ , regular, rigid, spiral depth 0.3 μ , regular. Waves, one or more gentle wavy curves throughout entire length. When in free space one or both ends may be semi-circularly hooked, while in semi-solid media the organism appears serpentine, waved or bent. The flexibility is most striking. Neither axial filament nor membrane recognized. Chambered structure, crista and flagella absent. Terminal finely spiral filament not recognized. Highly motile end portion well developed in the last six or eight spirals. Division transverse. Easily distinguished from *Treponema* and *Borrelia*. Filterable through Berkefeld V, N, and W candles.

Staining.—Membrane not recognizable. Body stains reddish by Giemsa solution.

Culture.—Grows under aerobic conditions at 25° to 30° C. on liquid or semi-solid media containing low concentrations of protein split products, serum and a trace of hemoglobin.

Resistance.—Complete to 10 per cent saponin.

Pathogenicity.—The natural animal reservoir is the rat. About 4 per cent of rats in New York found infected, in Japan 40 per cent. The leptospira are discharged in the urine and transmitted to man through contact with contaminated

Serology.—Recovery from an attack of Weil's disease usually confers lasting immunity. Antibodies including agglutinins and lysins are produced. Immune serum mixed with spirochetes and complement causes rapid lysis of the organisms.

Examination of Clinical Materials.—Specimens of blood, urine and serum should be examined. The spirochetes are most abundant in the blood during the initial period. Decreasing in the icteric stage they may not be detected late in convalescence. In the urine, however, the organisms are scarce early in the disease becoming more abundant during the icteric and convalescent stages. During the latter periods the lytic antibodies reach their highest concentration in the blood.

1. **Microscopic.**—Examine fresh preparations of blood and of centrifugized urine with darkfield illumination, also films stained with Wright's or Giemsa stains, for morphologically typical leptospira. They may be difficult to find.

2. **Culture.**—Inoculate blood and urine specimens into tubes of a suitable medium and incubate at 25° to 30° C. Larson* recommends Verwoort's medium, but states that Ringer's solution containing 5 to 10 per cent of serum (preferably human or rabbit) is perfectly satisfactory. The *Leptospira* grow best at the surface and in a zone extending about a centimeter below the surface where they often form a hazy, grayish ring.

3. **Serological.**—Larson recommends an adaptation of the Schüffner-Mochtar agglutination-lysis test for the demonstration of antibodies in a patient's serum against *Lept. icterohæmorrhagiæ*. The serum must be obtained and handled aseptically. It is diluted in sterile Wassermann tubes with sterile saline to provide a series of decimal dilutions extending from 1 to

one row of depressions on a sterile porcelain spot plate are mixed equal parts (0.1 cc.) of the serial serum dilutions and culture of *Lept. icterohæmorrhagiæ* to give final dilutions from 1 to 10 to 1 to 1,000,000. In a second row the same serum dilutions are similarly mixed with *Lept. canicola*, a form closely related to *Lept. icterohæmorrhagiæ*, found in dogs and sometimes in man.

The plate with its twelve spots per serum (plus suitable controls) is incubated at 32° C. in a large Petri dish containing wet filter paper to prevent evaporation from the 0.2 cc. of serum and culture in each spot. After four hours incubation each spot is examined. This is accomplished by transfer with a loop to slides on the darkfield microscope of a drop from the spot to be checked upon. Strict attention must be paid to two details: (1) No cover slip is applied to the drop to be examined; and (2) low power magnification is used. A positive test is characterized by the appearance of well-marked agglutination in the spots of greater serum concentration with lysis in one or more drops of next greater dilution and

* National Institute of Health, Bethesda, Md.

with, finally, neither lysis nor agglutination in the remaining drops of greatest dilution. The end point is the highest dilution in which there is lysis. Significant titres are 1 to 100 or over. Some workers use formalized cultures of *Leptospira*. In this case lysis does not take place and the end point is the highest dilution showing complete agglutination.

4. **Animal Inoculation.**—Inoculate blood and urine specimens subcutaneously or intraperitoneally into white guinea pigs. In the case of positive specimens fever usually develops within a day and persists until shortly before death, which occurs in four to ten days. The *leptospira* appear early in the blood, both extracellularly and within the leukocytes, and later in the urine. An intense jaundice develops late in the disease. After death the organisms can be found in the adrenals, liver and kidney. Protection tests are also made use of in which the unknown organism or serum is identified by the known serum or organism affording or obtaining specific protection in a susceptible animal.

THE SPIROCHETE OF RAT-BITE FEVER (SODOKU).

Sodoku or rat-bite fever is a spirochetal infection, first recognized in Japan, which occurs in many regions of the world. It should not be confused with Haverhill fever which is similarly transmitted and has been called rat-bite fever, but is associated with *Streptobacillus moniliformis*. There has been much confusion as to the classification of the spirochete which causes Sodoku, and it has been designated as *Spirocheta morsus muris*, *Borrelia muris* and *Spirillum minus*. In Bergey's Manual (1939) it is not included with the pathogenic spirochetes under the genera. *Borrelia*, *Treponema* or *Leptospira*, but is listed following the genus *Spirillum* of the Family *Pseudomonadaceæ*, as an "additional species mentioned in the literature." However, as indicated by Zinsser and Bayne-Jones (1939) *Spirillum minus* has many characters which justify its classification with the other disease-producing spirochetes.

Spirillum Minus

lesions in the mouth or infectious exudate may be a point of contact with pointed
crests and the long
am negative.
blood of an

In natural infections in man, the rat-bite wound usually heals; but, if left untreated, the wound becomes contaminated; after an incubation period of five to fourteen days or longer the wound swells, becoming painful and purplish. The swelling may become chancre-like, and the regional lymphatics and nodes become inflamed and tender. There is a relapsing type of fever and a characteristic rash. If untreated may last several months or longer. Death is rare in uncomplicated cases treated with arphen-amine or other suitable arsenicals.

Examination of Clinical Materials.—The specific diagnosis of Sodoku depends on the demonstration of *Sp. minus* in infected materials. Collect specimens of blood, exudate from the local lesions of the skin, serum from exanthematous patches or lymph nodes or ground up tissue excised from lesions.

1. **Microscopic.**—Examine the fresh material microscopically by dark-field illumination; also prepare and examine films stained with Wright's or Giemsa stains. The flagella can be stained best by the Tribondeau-Fontana silver stain.

2. **Cultural.**—No methods available.

3. **Serological.**—A so-called "immobilization test" may be used in which patient's serum mixed with animal blood containing spirilla causes the organism to lose motility. However, as this test is uncertain and often negative it is of value only as a confirmatory test.

4. **Animal Inoculation.**—Inoculate portions of the various specimens into at least 2 guinea pigs and 4 white rats. The injection should be made both subcutaneously over the abdomen and intracutaneously in the genital region. Observe the animals for infections, and examine lesions and blood microscopically for *Spirillum minus*.

OTHER BACTERIA

I. **The Genus *Lactobacillus*.**—Fifteen species of Gram-positive, micro-aerophilic to anaerobic bacilli belong to the genus *Lactobacillus*. They are non-motile, medium sized rods which ferment "carbohydrates," always producing lactic acid. *Lactobacilli* are able to withstand a degree of acidity usually fatal to non-sporulating bacteria. This characteristic may be made use of in their isolation as well as in their identification. They are often called the "aciduric group." Aciduric bacteria are of interest to us as possible incitants of dental caries, as important bacteria of the normal bowel and other body cavities, and because of their occasional use therapeutically. *Lact. bifidus* and *Lact. acidophilus* are found in the normal bowel. Döderlein's bacillus, a common constituent of the flora of the vagina, and the Boas-Oppler bacillus, from the gastric contents, are closely related to *Lact. acidophilus*. *Lact. bulgaricus* is an organism found in milk products. In many parts of Europe, Asia and Africa the natives use soured milk products, such as leben, yoghurt, matzoon and kefir. These products conserve milk and benefit health. They contain *Lactobacilli* and are the forerunners of modern *Acidophilus* therapy.

II. ***Alcaligenes Fæcalis*.**—Another bacterium ordinarily harmless which on occasion invades the body to produce disease is *Alc. fæcalis*. It is a Gram-negative form morphologically similar to *Eberthella typhosa*. It ferments no sugars and hence is easily distinguished from the *Enterobacteriaceæ*.

III. ***Chromobacterium Violaceum*.**—The three species of the genus *Chromobacterium* produce a violet, chromoparous pigment, soluble in alcohol but not in chloroform. These species are found in soil and water but *Chr. violaceum* is not infrequently encountered in abscesses and Soule has presented a careful study of two fatal human cases. Gram-negative. Motile. Gelatin liquefied. Indol —.

IV **Non-Spore-Forming Anaerobes.**—There is a large group of non-spore-forming anaerobic bacilli usually Gram-negative. Found normally

as inhabitants of the upper respiratory tract, genital tract and colon they may at times be associated with ulcerative processes and even invade the tissues and organs of the body producing abscesses, or the blood stream to give rise to septicemia. Generally neglected in routine bacteriological studies these bacteria may be present in "sterile pus" from surgically drained abscesses. Dack has reported that 3.86 per cent of 5180 specimens submitted for bacteriological examination in the Department of Surgery at Billings Memorial Hospital, University of Chicago, 1936-1940, contained non-sporulating anaerobes. More detailed information may be found in his monograph (1940). This group includes both cocci and bacilli which obviously bear a variety of names. They probably do not specifically cause any important disease condition but they participate in and complicate many inflammatory processes such as ulcerative stomatitis (see *Borrelia*), appendicitis, lung, pelvic and hepatic abscesses, pulmonary gangrene, puerperal sepsis, focal infections of the kidneys and chronic ulcerative colitis. Increased emphasis should be placed on anaerobic culture of clinical materials. This particular group is not particularly easy to study because they do not form spores and hence cannot be separated from non-spore-bearing aerobes simply by heating as can be done with the genus *Clostridium*.

V. *Listerella Monocytogenes*.—The genus *Listerella* containing one species is made up of small, non-spore-forming, Gram-positive rods, motile by means of a single terminal flagellum. They are aerobic to microaerophilic and grow readily on ordinary media. It has been suggested that this species is the cause of infectious mononucleosis. It does cause lesions in the organs, blood and cerebrospinal fluid of rabbits, sheep, cattle, fowls and gerbilles.

VI. *Streptobacillus Moniliformis*.—Among the "miscellaneous species of rod-shaped, non-spore-forming organisms whose taxonomic position is as yet undetermined" is *Streptobacillus moniliformis* regarded as identical with *Haverhillia multiformis* isolated by Parker and N. P. Hudson from an outbreak of "*erythema arthriticum epidemicum*" in Haverhill, Mass. The organism is a Gram-negative bacillus of variable size and form growing best under reduced oxygen tension and in the presence of blood or ascitic fluid. The organism has been found in mice and rats and it is possible that the "streptothrix" reported in cases of rat-bite fever may really be this species. Its pathogenicity for rats is doubtful but is found in these animals

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ciated with or give rise to another organism designated as "L₁" which possesses many properties in common with the microorganisms of pleuropneumonia and agalactia. For a full discussion of this point and of the pleuropneumonia group in general the monograph of Sabin (1941) should be consulted. The pleuropneumonia group is of considerable importance

The organ-
suitable solid
media may in two to seven days produce colonies which, under a hand lens because of the
for their demonstration.
relation of the "L" form

VII. *Bartonella Bacilliformis*.

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CHAPTER XXVII

BACTERIAL FOOD POISONING

By LELAND W. PARR

It has been generally recognized that the ingestion of foods contaminated with certain types of bacteria, or their products, may produce various clinical manifestations termed "Food Poisoning." There are three main types of this condition each associated with its own peculiar etiology. They are: (1) *Staphylococcus* food poisoning, characterized by symptoms of nausea, vomiting, diarrhea, and acute prostration coming on usually within two-and-a-half to three hours after eating. Toxigenic strains of staphylococci may grow rapidly in some food and elaborate enterotoxin within a few hours (five to twelve hours or longer) when suitable temperatures are provided. Such foods as milk, cured meats and cream-filled bakery goods are commonly involved. Uncomplicated staphylococcus food poisoning is not fatal. (2) Also caused by the ingestion of preformed toxin is Botulism which is characterized by difficulties in swallowing, double vision, difficulty in speech, labored breathing and finally, in a high proportion of cases, death from paralysis of the muscles of respiration. Symptoms appear within one to two days after eating the food containing the toxin which is usually improperly processed food, such as home-canned vegetables, in which *Clostridium botulinum* has grown.* (3) Salmonella infection of the food poisoning type is caused by the ingestion of large numbers of bacilli of this genus and presents a clinical picture in which abdominal pain, diarrhea, chills, fever, frequent vomiting and prostration are prominent features. Symptoms come on seven to seventy-two hours after swallowing the living organisms. The species most apt to be concerned are *Sal. enteritidis*, *Sal. choleraesuis*, and *Sal. typhimurium* (*Sal. ærtrycke*). Not ordinarily fatal.

In recent years a fourth type of food poisoning has been recognized, namely that caused by alpha-type streptococci. This form is less severe than the other types and is characterized by colic-like pains and diarrhea. Symptoms come on within a few hours after ingestion of hundreds of millions of bacteria, but are not of toxigenic etiology.

It must be remembered that the symptoms of food poisoning are not unlike those of a number of other conditions such as heart disease, brain tumor and the onset of infectious diseases. Symptoms coming on within a few minutes after eating are hardly likely to be bacterial food poisoning but whenever symptoms ensue two to three hours after eating food and particularly if several persons are involved a tentative diagnosis of food poisoning is in order. Dack has recently (1943) published a monograph on "Food Poisoning" which completely covers the subject. Table 73 from Dack summarizes the chief types.

* In the U. S. A. 367 outbreaks have been recorded between 1892 and 1942 of which 284 were caused by home-canned foods. In order of frequency of occurrence the foods were string beans, corn, beets, asparagus, pea foods, spinach and beans (listing only those implicated in 10 or more outbreaks).

TABLE 73.—CHARACTERISTICS OF FOOD POISONING CAUSED BY BACTERIA OR THEIR PRODUCTS (DACE'S FOOD POISONING, COURTESY OF UNIVERSITY OF CHICAGO PRESS)

Disease	Specific agent	In-toxi-cation	In-fec-tion	Symptoms	Onset of symptoms after eating
Botulism	<i>Clostridium botulinum</i> which produces toxin	+	—	Difficulty in swallowing, double vision (diplopia), difficulty in speech (aphonia), difficulty in respiration followed by death from paralysis of muscles of respiration	2 hrs to 8 days Average* 1-2 days
Staphylococcus food poisoning	Staphylococci which produce enterotoxin	+	—	Nausea, vomiting, diarrhea, and acute prostration, abdominal cramps	1-6 hrs. Average 2½-3 hrs.
Salmonella infection	<i>S. enteritidis</i> <i>S. choleraesuis</i>	—	+	Abdominal pain, diarrhea, chills, fever, frequent vomiting, prostration	7-72 hrs.
Streptococcus food poisoning	<i>S. erysipelae</i> a type	—	+	Nausea, sometimes vomiting, colicky pains, and diarrhea	5-18 hrs.

INVESTIGATION OF AN OUTBREAK OF FOOD POISONING

It is not always easy to determine the cause of an outbreak of food poisoning, and in many instances the source of the outbreak remains a mystery. In order to obtain the best results it is essential that the clinician, epidemiologist and bacteriologist work in close coöperation. The following general procedure is recommended for the investigation of an outbreak: (1) Prepare a list of all cases. (2) Obtain from each case a full history including information as to the various foods eaten prior to the symptoms. (3) If possible determine the foods responsible for the illness. (4) Study the history of the implicated food. (5) Try to determine the source from which the food was contaminated.

The materials to be collected for examination include: (1) Left-over portions of the suspected foods. (2) Specimens of vomitus, feces and blood serum from the cases, and at a later date, additional specimens of serum for agglutination tests. (3) Feces and blood from food-handlers or others suspected as carriers. (4) Specimens of blood, spleen, liver and intestinal contents from fatal cases. The specimens of food should be immediately packed in ice and examined with the least possible delay.

I. Bacteriological Examination.—The bacteriological examination includes search for the organisms mentioned above or for bacterial toxins, and may be carried out as follows.

1. First Day.—(a) Prepare Gram-stained films from liquid portions of the foodstuffs, or from suspensions of solid foods, and examine these microscopically to determine the morphological features of the predominating organisms.

(b) Prepare cultures on eosin-methylene blue (or S.S. agar) plates for the detection and isolation of organisms of the genus *Salmonella*; infusion agar and broth for staphylococci and on anaerobic media for *Cl. botulinum* if this organism is suspected. In the latter case inoculate several tubes of Robertson's medium or Brewer's thioglycollate. Half the tubes may be heated to 70° C. for twenty minutes and incubated at 35° C.

(c) *Tests for Toxin.*—Samples of the suspected foods may be tested for preformed toxins or for pathogenic bacteria by oral or subcutaneous administration to guinea pigs or white mice in the case of botulism or to cats or suckling pigs as has already been indicated in the section on Staphylococci (see page 449).

When *Cl. botulinum* is suspected prepare a suspension of the food in physiological saline, centrifugalize or filter until clear and inject about 1 cc. subcutaneously into each of 3 guinea pigs. At the same time inject intra-

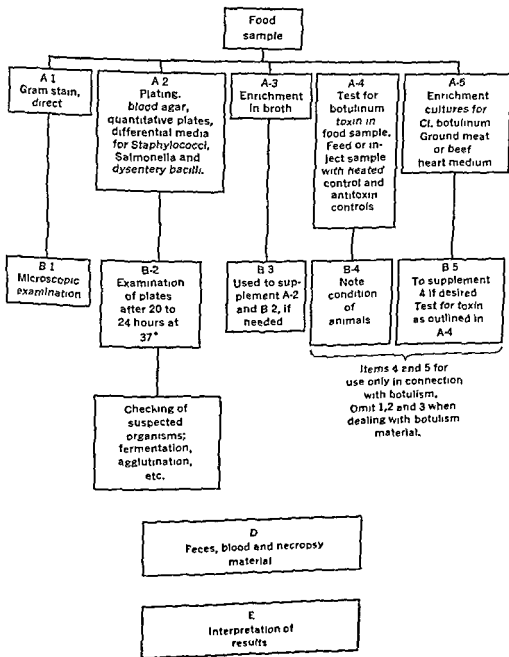


FIG. 42 — Outline of procedure for examination of a food sample (Adapted from Diagnostic Procedures and Reagents, courtesy of Am. Pub. Health Association)

peritoneally into the first animal 1 cc. of Type A *Cl. botulinum* antitoxin, into the second a like amount of Type B antitoxin and into the third pig the same amounts of both the A and B antitoxins. If *Cl. botulinum* toxin is present in the food, the type can be determined as indicated below.

TEST FOR TYPE OF BOTULINUM TOXIN PRESENT

Guinea pigs protected with antitoxin	<i>Cl. botulinum</i> toxin subcutaneously		
	A	B	A and B
Pig 1 (Type A)	Live	Die	Die
Pig 2 (Type B)	Die	Live	Die
Pig 3 (Types A and B)	Live	Live	Live

If Pig 1 dies it is Type B, if Pig 2 dies it is Type A, if both die it is a mixture of Types A and B, provided Pig 3 lives. If no pigs die *botulinum* toxin is not present. If all three die it is *botulinum E* or some other lethal factor which killed them.

There are, of course, other types of *botulinum* toxin but Types C and D have not been reported as causing human outbreaks of botulism and Type E is rather rare. Its antitoxin can be included with the two commonly used if it is desired to search for that type.

2. **Second Day.**—Examine plates for staphylococci and for members of the *Salmonella* group. If the latter are present isolate pure cultures and identify by the methods outlined under *Salmonella*. Note the presence of any other type of organism. If the anaerobic cultures contain growth, examine them microscopically for organisms with the morphological characteristics of *Cl. botulinum*, and if such are found subcultures may be made for pure culture study and test for toxin. A diagram of the procedures suggested is shown in Figure 42.

CHAPTER XXVIII

BACTERIOLOGIC EXAMINATION OF MILK AND OTHER DAIRY PRODUCTS

By LELAND W. PARR

THE primary purpose for which milk and other dairy products are examined bacteriologically in Army medical laboratories is to determine their suitability for consumption by military personnel. Estimates of the total numbers of bacteria present in such products and special procedures for the identification of pathogenic organisms serve to indicate they are potable and safe for human use. The tests used are those recommended in *Standard Methods for the Examination of Dairy Products*, 8th Edition, published in 1941 by the American Public Health Association.

MILK

I. Specimens.—To determine whether new milk supplies conform to sanitary regulations, examine not less than four samples each taken on a different day. The specimens should be carefully collected under aseptic conditions and should be representative of the lot from which taken. Samples of large quantities of milk should be collected at the dairy farm and samples from the large cans may be taken as they are delivered at the pasteurizing plant. The examination of such specimens furnishes an index to the efficiency of methods used to prevent contamination. Samples of bottled milk may be selected at random either from the stock on hand at the plant, or from milk delivered for sale and consumption. After thoroughly mixing the milk in its container, collect the specimen with a sterile pipet long enough to reach the bottom of the can or bottle. Each specimen must include at least 10 cc., and should be kept iced in a tightly stoppered sterile bottle or vial. If the examination cannot be made within four hours after collection, the exact time elapsing should be reported.

II. Enumeration of Bacteria.—1. **Standard Plate Count.**—After shaking the milk specimen twenty-five times, with an up and down excursion of about 1 foot, total shaking time seven seconds or less, prepare dilutions of about 1 to 100, 1 to 1000, and 1 to 10,000. What is known as the standard plate count is made by transferring 1 cc. amounts from each to properly labeled sterile Petri plates. Within twenty minutes time from the first transfer from the sample add to each plate 10 to 12 cc. of standard nutrient agar which has been melted and cooled to 40° to 45° C. In milk work this is a tryptone-glucose-extract-milk agar of preferred reaction pH 7.0. The agar contains 1 per cent skim milk as a part of the medium when samples diluted more than 1 to 10 are used. Mix the agar and sample thoroughly, solidify quickly and incubate at once. Two temperatures of incubation are recognized as standard: (a) at 32° C.; and (b) at 37° C., both for forty-eight hours. Following incubation examine the plates with a lens magnifying 2½ diameters (a Quebec Colony Counter will be found most useful) and count the

colonies on plates showing between 30 and 300 each. If none of the plates show a number within these limits the one having nearest to 300 should be counted. Determine the average count and report the result as: The number of colonies per cc., or as the "standard plate count" number per cc. The temperature of incubation should also be stated whether 32° or 37° C. The total number of bacteria present in the milk is always greater than the count shows because there are bacteria present which may not grow into colonies because the temperature of incubation is too high (true psychrophiles, which grow slowly at 5° to 10° C.), because the temperature is too low (true thermophiles, which grow at 55° C.), or because the medium used and the aerobic type of incubation employed will not permit them to develop (fastidious pathogens, obligate anaerobes and the like). It is customary to prepare two plates per sample, on each of the two dilutions most likely to yield satisfactory plates. Duplicate plates are not considered necessary.

2. *Microscopic Count of Bacteria (Breed Method).*—Exactly 0.01 cc. of the milk to be examined is drawn up into a special capillary pipet and is spread uniformly over an area of 1 sq. cm. on a microscopic slide. Dry the film in a warm place for not more than five to ten minutes, avoiding excessive heat. Immerse in xylene one minute to remove fat, drain and allow to dry. Fix for one minute in 90 per cent alcohol. Stain with Loeffler's methylene blue solution, rinse with water, decolorize with alcohol until only a faint blue tint is left; then dry and examine microscopically.

The number of bacteria per cc. of milk is estimated by counting all the organisms within a given area in a microscopic field, this area having been carefully measured and its ratio to a square centimeter determined. At least 1/100,000 part of a cc. of milk is to be examined, and if the milk is of high grade this must be done under the most favorable conditions for accurate counting. The microscope must be so adjusted that each field covers a certain known fraction of the area of a square centimeter. This adjustment is simple if a micrometer slide, ruled in hundredths of a millimeter is at hand. The microscope should have a 1.9 mm. (1/12 inch) oil-immersion lens, and an ocular giving approximately the field desired (for example a 6.4 X ocular), and it should be fitted with a mechanical stage. To standardize the microscope, place the micrometer slide on the stage and by the selection of oculars or by adjustment of the draw tube, or both, bring the diameter of the whole microscopic field to exactly 0.205 mm. When so adjusted, each field of the microscope covers an area of approximately 1/3000 sq. cm. (actually 1/3028 sq. cm.). This means that the dried milk solids from 1/300,000 part of a cc. of milk are visible in each field of the microscope. Therefore, if the bacteria in one field only are counted, the number found should be multiplied by 300,000 to give the estimated number of bacteria per cc. In practice, however, more than a single field is examined so that the factor used for multiplication is smaller than this.

As the microscopic examinations must be made with greater care when the bacteria are relatively few in number, it is required that, in grading low count milk, a special ocular micrometer with a circular ruling divided into quadrants shall be used. In using this micrometer, the microscope shall be so adjusted that the diameter of the circle on the eye-piece micrometer shall be 0.146 mm. In this case the amount of dried milk solids examined in each field of the microscope is 1/600,000 part of a cc. of milk. The

limitation of the examination to the central portion of each field obviates the use of the margins of the field where definition may be hazy, and lessens the danger of overlooking bacteria. Likewise, the magnification used is greater than that used when the whole field is to be examined.

The microscopic method of estimating the number of bacteria in milk has the following advantages: (1) It makes possible the counting of all bacteria, living or dead, and can therefore be used on specimens preserved with formalin or other antiseptics. (2) It is more economical and can be carried out rapidly in the field. (3) It gives information concerning the sanitary condition of the dairy and the contaminated milk before pasteurization. The disadvantages of the method include the following: (1) The small amounts of milk used lead to inaccuracy and the large factors used in estimating the bacterial count introduce a large degree of error. (2) Much time must be spent on the counts in order to reduce this degree of error, especially when examining very good milk. (3) The individual technic of the counter may be responsible for a greater variation in results than when the plate method is used. The ratio used in comparing the standard plate count with the microscopic bacterial count is computed at 1 to 4. When estimating the probable standard plate count by the microscopic method, clumps of bacteria should be counted as individual units.

3. Count Interpretations and Milk Designations.—(1) Raw milk is untreated (except for refrigeration) milk. (2) Pasteurized milk is milk that has been treated with limited heat by one of several methods, in order to kill most pathogenic bacteria. (3) Certified milk is an especially pure raw or pasteurized milk generally for infant feeding, produced under the supervision of a medical milk commission of the county or state Medical Society, based on requirements of the American Association of Medical Milk Commissions. Certified raw milk should not present a standard colony count to exceed 10,000 per cc. and certified pasteurized milk is prepared from raw milk of certified quality and which after pasteurization does not yield more than 500 colonies per cc.

The Standard Milk Ordinance and Code of the United States Public Health Service (1939) classifies and defines milk as: (a) Grades A, B, and C, raw; (b) Grades A, B, and C, pasteurized.

(4) Grade A pasteurized milk is the grade usually sold for drinking purposes; must have a colony count of not over 30,000 per cc. and must be prepared from Grade A (50,000 per cc.), or Grade B (200,000 per cc.) raw milk in plants meeting strict sanitary requirements.

Other grades of milk are based on definite sanitary requirements for the production, distribution and bacterial content. The allowable colony counts for raw milks are greater than for the corresponding grade of pasteurized milk; also, the sanitary requirements are progressively less rigid and the allowable colony counts greater for grades B and C milk, respectively. Federal regulations specify that all fluid milk purchased by the government shall conform to the Standard Milk Ordinance and Code standards.

III. Methylene Blue Reduction Method.—This test, which is known as the reductase test, is useful where laboratory facilities are limited, or for making a rapid inspection of a large number of samples. It is based on the fact that when methylene blue is added to milk the color may be

end. Acetylsulfathiazole crystals sometimes resemble those of the free drug but the angle at the ends is 136 degrees. More commonly they have the appearance of sheaves of wheat with a central binding. These may present any degree of fullness up to a stage where they form two half circles fused at the center. Striated spherulites are also quite common.

(4) *Sulfadiazine*.—Both the free and acetyl forms appear, the former perhaps more frequently as it is the less soluble form. The free drug forms dense dark greenish irregularly striated spheres with either fuzzy or clean edges. Acetylsulfadiazine crystals form sheaves of wheat with eccentric bindings. They are easily differentiated from acetylsulfathiazole.

(5) *Sulfaguanidine*.—The free form rarely appears. Crystals of acetylsulfaguanidine are thin oblong plates, clear or with a fine mesh-like pattern, often aggregated into cross or star-like clusters.

(6) *Sulfasuccidine* crystals do not appear in the urine because of the slight absorption of the drug from the intestine.

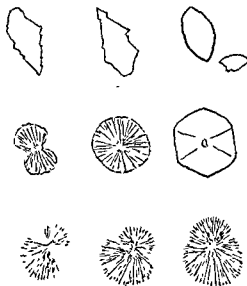


Fig. 2.—Crystals appearing in human urine after administration of *Sulfadiazine*.

These figures are reproduced from micro-photographs of urinary sediment, enlargement 250X (Lehr and Antopol, courtesy of Science)

2. **Organized Sediments.**—These structures in the urinary sediment are of the greatest importance. Their proper recognition and evaluation may often be the most important part of the examination of the urine. Accurate identification may necessitate the use of the high-power of the microscope particularly in cases of doubt.

(a) **Epithelial cells** may appear in the urine.

which the cells are derived. be observed.

(1) Small round or polyhedral cells with a single round rather large nucleus. These probably come from the kidney tubules.

(2) Transitional cells from the bladder, ureters and pelvis of the kidney are often larger than the round cells, have smaller nuclei and may be of various shapes—pear-shaped, spindle-shaped or round.

(3) Squamous or pavement cells are the most frequent. They are large, flat, irregularly shaped and have small nuclei. In urine from women they are particularly abundant and represent vaginal contamination.

(4) Leucocytes, White Blood Cells or Pus Cells.—These are the round mononuclear or polymorphonuclear white cells of the blood, most of them being the familiar polymorphonuclear leucocytes. They may occur singly or in clumps and are granular in appearance. The addition of a little dilute acetic acid to the slide brings out the nuclei and may help identification. A few of these cells are usually present. Any increase indicates an inflammatory process. The site of the inflammation may often be determined by means of the accompanying structures or by the clinical symptoms of the patient.

(5) Erythrocytes are the red cells of the blood which have gotten into the urine. In fresh urine they appear as biconcave discs. They become swollen and rounded in dilute and crenated in concentrated acid urine. In alkaline urine they are apt to become faint, colorless, disintegrating shadows. Identification should be by means of the high-power lens so as to avoid confusion with fat, yeasts or oxalate crystals. Their presence can be confirmed by chemical tests, though when present in small numbers chemical tests are apt to be negative.

Red blood cells in small numbers do not alter the appearance of the urine. Large numbers cause a smoky or frankly blood-tinged appearance. Their presence is always of pathological significance, indicating bleeding somewhere in the urinary tract.

(6) Casts.—These structures are casts of the urinary tubules. They are composed of an albuminous material, degenerated renal tubule cells, red blood cells and pus cells that have plugged the tubules in the case of inflammation or hemorrhage. They are probably always indicative of pathologic change in the kidney though this may be transitory in nature. Casts may be straight or curved, long or short, narrow or thick. Their sides are usually sharply outlined and parallel, their diameter constant throughout their length, their ends rounded or broken off straight across. They should be sought under low-power magnification and low illumination.

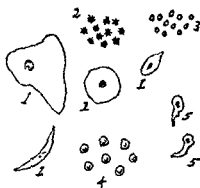
(1) Hyaline casts are the most frequent. They are colorless, homogeneous, non-refractile, semitransparent structures. They are usually narrow but may vary considerably in size and shape. Rather frequently a few cellular elements or granules may adhere to their surfaces. They are generally of less import than the other types of casts.

(2) Granular casts are usually shorter and thicker. Their surface is studded with fine or coarse granules, the latter often being dark brown in color. The coarse casts are usually associated with more severe renal lesions.

(3) Waxy casts are homogeneous, highly refractile, sharply outlined, light yellow, opaque, dull, short and thick. The ends are usually broken and the casts sometimes appear to be segmented. They are rather rare and as a rule appear only in the advanced stages of nephritis.

(4) Cellular casts are of many types, depending upon the type of cell embedded in its surface. They are identified by the type of cell found—

epithelial, pus, blood or bacterial. Such casts usually indicate acute processes or late stages of chronic disease.



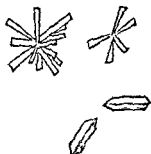
1, 5, Epithelial Cells
2, 3, Erythrocytes
4, Leucocytes



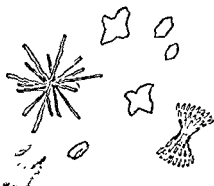
1 & 2 Triple Phosphate Crystals



1, Leucocytes; 2, Spermatozoa;
3, 4, 6, Epithelial Cells;
5, Erythrocytes



Neutral Calcium Phosphate
Crystals



Uric Acid Crystals. (Yellow
to Greenish Yellow.)



Uric Acid Crystals. (Yellow
to Greenish Yellow.)

FIG 3 — Urinary sediments. (By courtesy of the Medical Dept., United States Army)

(5) Cylindroids resemble hyaline casts. They differ in that they taper to a point or a slender tail. Threads of mucus are commonly mistaken for cylindroids. True cylindroids have much the same significance as hyaline casts.

(e) Mucus.—Threads of mucus may simulate casts or cylindroids. They differ, however, in appearing as long strands, ribbon-like in character with poorly defined edges. They are a normal part of the urine and are of no significance.

(f) Bacteria appearing in the voided urine are usually contaminants from the urethra or vessels containing the urine. No attempt is ordinarily made to identify them. Catheterized specimens may be examined by the usual bacteriological methods.

(g) Spermatozoa occasionally are found. The characteristic oval head and long tail makes for ready identification. In fresh urine they may still be motile.

(h) Animal parasites in the urine are rare. Cysts and hooklets of echinococcus, ova and occasionally the adults of *Schistosoma*, filarial organisms and larvæ have been reported. *Trichomonas* and *cercomonas* may be seen particularly in the urine of females. The ova of intestinal parasites such as *ascaris* and *oxyuris* may contaminate the urine.

(i) Confusing structures superficially resembling important findings may sometimes lead to difficulty. They must be properly identified in order that reports may be accurate.

(1) Yeast cells are smooth, colorless, highly refractile, round or oval structures. They may be mistaken for erythrocytes. However, they are of irregular size, tend to adhere in short chains and often have buds at their edges.

(2) Mold fungi have jointed or branched rods often arranged in a network. Highly refractile spherical or oval spores may be present.

(3) Fibers of cotton or wool, bubbles of air, dirt and flaws or scratches in the glass slide should not be confusing if care is exercised in the examination.

IV. Quantitative Microscopic Method.—The Addis Count.—Addis has developed a method for determining the numbers of red blood cells and casts appearing in the urine under standardized conditions. By this technic accurate results are obtained and better clinical interpretations are possible.

1. *Specimen.*—All of the urine voided from 6 P.M. to 6 A.M. is collected after an all-day water fast on a dry diet. The urine is collected in a thoroughly cleansed, wide-mouthed bottle rinsed with 4 per cent formaldehyde solution before the collection begins.

2. *Procedure.*—The concentrated night urine is thoroughly mixed and the volume measured. Ten cubic centimeters of the mixed specimen, which has been cleared of excessive phosphates by the addition of weak acetic acid

shows varying shades of red or brown in hemorrhage, but with very slight bleeding, the precipitate is white. The greater part of the supernatant fluid is then decanted and the residual urine removed with a pipet and aspirating bulb, leaving 0.5 cc. of a mixture of urine and sediment, or a volume varying with the amount and nature of the deposit. This is thoroughly mixed. A small drop is transferred to a hemocytometer. Using a 16 mm. objective and a 10× eyepiece, all the cells and casts are

counted in the nine squares. In normal urines casts are very few, so 10 drops are counted. If casts are numerous, dilute the sediment in the proportion of 1 to 5 and count only 2 drops. The differential cast count is made at the same time.

3. *Calculation.*—As all 9 squares of the hemocytometer are counted, each being 1 sq. mm. in area, and the chamber 0.1 mm. deep, the total volume of sediment examined is 0.9 c. mm. or 0.0009 cc. If 10 drops are examined, the total volume is 0.009 cc. Suppose that in this volume 180 casts were found and the volume of sediment from which the drops were taken was 0.6 cc. Accordingly, $180 \times \frac{0.6}{0.009} = 12,000$ casts in 0.6 cc. But all the casts in 10 cc. of urine were concentrated in that 0.6 cc. of sediment, so 12,000 casts were present in the 10 cc. of urine. If the total volume of urine for the twelve hours was 300 cc., then $12,000 \times \frac{300}{10} = 360,000$ casts in the twelve-hour specimen. A similar calculation is employed for red blood cells. The general formula is:

$$\frac{\text{Number casts or cells counted}}{\text{cc. sediment examined}} \times \frac{\text{cc. total sediment}}{\text{cc. urine centrifugalized}} = \frac{\text{casts or cells in 12-hour urine}}{\text{cc. urine centrifugalized}}$$

Applying this formula to the above example the entire calculation is:

$$180 \times \frac{0.6}{0.009} \times \frac{300}{10} = 360,000 \text{ casts in the twelve-hour urine.}$$

4. *Interpretation.*—Highly concentrated urine is almost an isotonic medium for erythrocytes, thus preserving them. The small amount of formalin in the collecting bottle aids in preserving red blood cells and casts. This method will detect microscopic renal bleeding which the examination of a single dilute specimen misses. There is a wide variation in the normal counts of casts, red blood cells, epithelial cells and white blood cells. In adults casts range from 0 to 4220; red blood cells from 0 to 425,000; epithelial and white blood cells from 9000 to 2,823,000. Among normal adults practically all of the casts are hyaline; 73 per cent of adults give zero counts. Normal adults may excrete small numbers of red blood cells; about 60 per cent give zero counts. In comparing the number of casts found with the qualitative reports on the same urine, counts of 1000 to 79,000 give negative microscopic reports when examined in the routine manner. With counts between 122,000 and 1,000,000, the routine microscopic examination shows 1 or 2 casts per low-power field.

CHAPTER II

KIDNEY FUNCTION TESTS

By CLEON J. GENTZKOW and HOWARD A. VAN AUKEN

THE kidneys through their excretory function serve several purposes: the elimination of most of the waste products of the metabolic processes of the organism; the excretion of harmful substances which may have been ingested; and control of the osmotic, electrolyte and acid-base balance of the blood and tissue fluids. It is through these processes that the constancy of the fluids, in which the cells are bathed, is maintained, a constancy which must be maintained within very narrow limits if life is to continue.

The structural and functional unit of the kidney is the nephron made up of a glomerulus and a corresponding tubule. The number of nephrons is estimated to be about 2,000,000. They do not all function continually, the number in action appearing to vary with the excretory demands upon the kidney. This fact may serve to explain, in part at least, the great reserve capacity of the kidneys. One kidney may be congenitally absent or surgically removed, and yet no apparent clinical evidence of renal embarrassment be apparent.

The manner by which the nephron excretes urine is becoming rather well understood. The development of the present theory of urine secretion has been gradual and continuous, many workers contributing to its understanding. Bowman, in 1842, considered that the fluid portion was secreted by the glomerulus, to which the tubules added the solid portions, the entire process being one of vital activity. Ludwig proposed the first filtration-reabsorption theory when he suggested a process of simple physical filtration of the water with its dissolved crystalloids through the glomerulus, the filtered fluid passing down the tubule where it was concentrated by reabsorption of water. Cushny, realizing that there were differences in the blood and urine concentration ratios of the various solutes, accepted glomerular filtration, but suggested a selective reabsorption of the solutes as well as of water. He introduced the terms, "no-threshold" for those substances which are excreted whenever present in the blood, and "thresh-

the glomerular fluid is a true filtrate with urea, uric acid, chloride, creatinine, glucose and pH in approximately the same concentration as in the plasma. Also, by tapping the tubules at various levels, he showed the points in the tubules where the various solutes were reabsorbed. Smith and Rehberg using inulin and creatinine respectively, measured the glomerular filtrate and arrived at values in close agreement. Van Slyke and Rhoads, using a different approach, confirmed these values and also showed that about 20 per cent of the blood flowing through the kidney was filtered in the glomeruli. Shannon studied the reabsorption of glucose and showed that it behaved in the same manner as a number of reactions of enzymes with their substrates. Marshall has proved that some foreign substances

introduced into the blood stream, notably phenol red, are excreted by the tubules.

From the work of the above investigators, as well as that of others, the following explanation of the functioning of the kidney may be drawn. About 20 per cent of the water with its contained crystalloids is filtered from the blood by the glomeruli, this filtration being a mechanical process brought about by the high pressure of the blood in the glomeruli. During the passage of the fluid down the first part of the tubules, 90 per cent of the filtered water returns to the blood because of the difference between the osmotic pressures of the two fluids and the drop in blood pressure in the capillaries about the tubules. Glucose and other threshold substances appear to combine with a hypothetical carrier substance in the tubule cells and are passed back to the blood stream. These carrier substances can function at a definite speed; when the concentration of the solute in question exceeds the particular level some of the threshold substance will not be reabsorbed and will thus appear in the urine. During the rapid back diffusion of the water some substances, such as urea and uric acid, are swept back, the tubules being only partially impermeable to these solutes. Creatinine is held entirely in the tubular concentrate. In the distal parts of the tubules more water is reabsorbed which results in a marked degree of concentration. Urea now reenters the blood stream by passive diffusion as a result of its increased concentration in the fluid. During the passage of the fluid through the tubules certain foreign substances that cannot be filtered through the glomeruli are excreted by the tubular cells. This tubular excretion comes into operation, however, only when such materials are present in the blood. With the exception of the reabsorption of the threshold substances and the last portion of the water, kidney excretion is a mechanical process of filtration and diffusion. Further explanation of those processes is needed.

The normal kidney is capable of varying the composition of the urine within wide limits to meet the needs of the body. It can excrete an excess of water promptly, resulting in a dilute urine; or it can withhold water in conditions of dehydration and eliminate a highly concentrated urine. Thus the specific gravity may range from 1.002 or lower, to 1.026 or higher. In progressive impairment of renal function the maximum gravity attainable falls and approaches 1.010; at the same time the minimum gravity rises toward 1.010. In the later stages of nephritis the specific gravity is apt to be fixed between 1.008 and 1.012. This is practically isotonic with the blood serum; the severely damaged kidney can no longer alter the glomerular filtrate presented to it.

In like manner the healthy kidney will excrete promptly the nitrogenous waste products in the blood. There are insufficient gl... as fast as it is added by the n... substances eventually accumulate in the blood stream.

The kidney also maintains the reaction of the blood and through the blood the reaction of all of the body fluids. This is accomplished by selective reabsorption of bicarbonate and the production of an acid or an alkaline urine. Ammonia is formed from urea and serves to neutralize acid when there is a marked excess. Both of these functions become decreased in renal disease and, as a result, in the late stages of nephritis acidosis fre-

quently develops. Finally there is a progressive loss in the active tubular excretion of foreign substances as kidney function fails.

Kidney function tests are all based on the measurement of one or more of these renal functions. It must be emphasized, as pointed out by Fishberg, that the kidney does not possess a number of functions which are independent of one another. Injury to one part of the kidney is reflected by changes in the remainder of the organ and when impairment of renal function develops, the excretion of all urinary constituents is affected. Fishberg maintains that selective retention is extra-renal in origin and explains the apparently selective injury on a purely quantitative basis. Irrespective of the type of injury that affects the kidney—be it inflammatory, arteriosclerotic, toxic, obstructive, multicystic or degenerative—the impairment is the loss of concentrating power, the impairment of clearance, the blood.

Fishberg also differentiates between renal insufficiency and impairment of renal function. In both there is a loss of concentrating power. In the latter, by means of a compensatory polyuria, that is, increased excretion of water, the waste products are excreted without damming back in the blood stream. In the former, there is no polyuria to maintain the balance, and there is retention in the blood of those substances normally highly concentrated by the kidney. When fully compensated, only the lowering of the maximum concentration evidences the hypofunction present; when decompensated there is a low maximum specific gravity, oliguria, low dye output and an increase in the nitrogenous constituents of the blood.

The results of any kidney function test must be interpreted in view of the entire clinical background of the patient. The tests measure function only, and not the extent of anatomic damage. It is well known that kidney function may be markedly decreased by many extra-renal conditions, such as cardiac decompensation, severe anemia, continued vomiting, prostatic obstruction, starvation and infections and inflammation of the lower urinary tract. The results of renal function tests should not, therefore, assume the form of a diagnosis; they should be viewed as a part of the complete study of the patient.

TYPES OF TESTS AVAILABLE

Of all the tests of renal function that have been devised only a few are necessary for investigation of the efficiency of the kidneys. Many of the published tests are but slight modifications of others; improved methods have supplanted many of the earlier tests. Only those tests combining the maximum of information and ease of performance need be carried out. Some types are undesirable because of technical difficulties in their performance. The studies may involve investigation of the urine, the blood or both. They may conveniently be grouped together under a number of heads according to type

I. Tests based on determination of the concentrating power of the kidney—"Concentration Tests."

II. Tests based on the excretion of foreign substances—"Dye Tests."

III. Tests based on the removal of nitrogenous constituents from the blood—"Clearance Tests."

IV. Tests based on determination of the constituents of the blood— "Blood Nitrogen Determinations."

The concentration tests are perhaps the most sensitive in detecting early renal impairment. In many instances if the concentration is normal, no further studies will be necessary. The dye tests and clearance studies are of the greatest value in following the later stages of nephritis already established. The dye tests alone are generally used when investigating the function of the two kidneys separately. Nitrogen retention tests are of value only in the latest phases of disease. They may indicate further changes when other tests already show maximal impairment. In every case, careful routine examination of the urine particularly for albumin, specific gravity, casts and red blood cells must be done at frequent intervals.

CONCENTRATION TESTS

According to Van Slyke and Fishberg these tests indicate renal impairment earlier than any others. They are especially useful as they cause but little inconvenience to the patient or doctor. They will indicate impairment of function, whether it is in the compensated or decompensated phase, but will not differentiate these phases, nor differentiate the various types of renal damage. When healing occurs in acute nephritis, the concentrating power is the last function to return to normal. It is, therefore, the test of choice for following those cases which are apparently clearing completely.

I. Fishberg Method.—This is a simple and excellent test; if supplemented by determinations of nitrogen in the blood it will show the presence or absence of renal impairment and whether any hypofunction present is in the compensated or decompensated phase.

1. Procedure.—A period of thirst is started at lunch, or more commonly at supper the day before the test. About 6 P.M. the patient eats his usual supper except that the fluids are minimal, no more than 200 cc. This meal should have a high protein content. After this no fluid or food is taken until the test period is completed. At bedtime the bladder is emptied. This urine and any other urine passed during the night is discarded. On awakening in the morning or at 8 A.M., urine is passed and saved. The patient remains in bed and again passes urine at the end of an hour from the previous voiding. He can then arise or stay in bed. Another and final specimen is passed one hour from the second. Each specimen is kept separate and labeled with the time of voiding. The specific gravity of each specimen is taken accurately, making corrections for temperature and albumin content if necessary.

2. Results.—If the kidneys are normal, one specimen will exceed 1.022, after at least one hour. In young persons the kidneys concentrate as well as the young. In old persons the maximal gravity is 1.010. In true uremia the maximum gravity lies between 1.010 and 1.020, usually nearer the lower figure. In patients with edema which is believed to be due to renal impairment, the specific gravity is usually low. In patients with nocturia, is no concentration; in these the specific gravity is higher during the day. The specific gravity of the first specimen is often the highest.

II. Lashmet and Newburgh Improved Method.—If the kidneys are loaded to capacity the specific gravity of urine should measure the renal power to excrete all the urinary constituents. The test seeks to present to the kidneys precisely the same amounts and kinds of products in a unit of time.

1. Procedure.—About 6 P.M. the patient eats his usual supper, avoiding all fluids. No food or drink is taken until the test is finished the next day. At bedtime the urine is voided and discarded. On waking the following morning the urine is voided, saved and marked "No. 1." Still in bed and one hour later, a second specimen is obtained. A third specimen may be taken if the patient awakes earlier than 7 A.M. The specific gravity of the 2 or 3 specimens is taken. An accurate urinometer should be used, correcting for temperature if it is more than 2° on either side of that at which the urinometer is standardized. Examine the sediment microscopically for casts, red blood cells and pus. If the gravity is 1.025 or less, question the patient as to the exact following of the instructions. To check abnormal gravities below 1.026, give a dry diet for one day. The diet should consist of: Breakfast: cornflakes, $\frac{1}{2}$ cup; bread, 2 slices; sweet butter, 2 squares; rich cream, $\frac{1}{4}$ cup, sugar 1 tablespoonful. Lunch: beefsteak; 1 small baked potato; crackers, 4; sweet butter, 2 squares; dates, 10. Dinner: boiled potato, 1; lettuce $\frac{1}{4}$ head; crackers, 4; sweet butter, 2 squares, dates, 4; peach, canned (no juice), $\frac{1}{4}$; 1 gm. salt is used in cooking. Beginning day of dry diet, no other food or drink all the urine from 8 P.M. until 8 A.M. the following morning. No breakfast is taken on this day. A second specimen is passed at 10 A.M., and a final one at noon.

2. Results.—This prolonged water fast will sometimes show normal concentrating power when the dry supper and overnight water fast fail. Normally the gravity is 1.029 to 1.032. Cases of essential hypertension have a gravity of 1.025 to 1.029. In Bright's disease the maximum ranges from 1.010 to 1.029, the lower the reading the more severe the disease. The maximum gravity is usually 1.015 before there is an increase in blood urea. In heart failure the gravity tends to be constant around 1.020, but the twenty-four hour volume is markedly lessened.

III. Dilution or Water Test.—It is better to perform this test independently of the concentration test. Preferably keep the patient in bed. Many patients find it difficult to take the large amount of water required. The test places a strain on the water-excreting function of the kidneys. It should not be performed in the presence of edema or of a failing circulation.

1. Procedure.—Early in the morning the patient voids completely; this specimen is discarded. Breakfast is omitted. Instead he drinks 1200 cc. of water in twenty to thirty minutes. For lunch and dinner a dry diet is given, or some prefer to give the routine nephritic diet or the usual diet to which the patient is accustomed. The collection of the urine specimen varies with different modifications of the test. Volhard and Fahr have the patient empty the bladder every half hour until noon, beginning at 8.30 A.M., the water having been given at 8 A.M. In their method all the urine from 12 noon, on the day of the test, to 8 A.M. the following morning is saved as one specimen. Nicholson allows the patient to voluntarily pass his urine when he desires, saving a sample from each measured specimen passed

during the day. Others collect hourly specimens from 8 A.M. until noon. In any case the volume of each specimen is noted and the specific gravity taken. In the half hour method of collection a curve is plotted.

2. **Results.**—The normal individual eliminates approximately 1200 cc. in four hours, the larger part in the first two hours. The specific gravity of at least one specimen should be as low as 1.002 to 1.003 and is usually the largest of the hourly specimens. The quantity excreted varies between 80 and 120 per cent of the amount taken. Later in the day the amount decreases and by late afternoon the gravity will rise as high as 1.025.

If the water elimination is impaired, the quantity eliminated in four hours will be small, sometimes less than 200 cc., and the specific gravity may not go below 1.010 or above. The cause of decreased excretion may be renal, or extra-renal as in edema or cardiac failure. The differentiation between a renal or extra-renal etiology may be made by a concentration test, for if the concentrating power is unimpaired the cause must be extra-renal. But if the concentration test shows a lowered concentrating ability, the cause can again be renal or extra-renal.

The ability of the kidney to excrete water may be impaired in any type of renal disease and is often most pronounced in nephritis associated with oliguria and edema. A polyuria may mask a deficient water excretion; even if only 400 cc. are eliminated in four hours, 2400 cc. could be excreted in twenty-four hours.

Normally the variation in the volume of the individual specimens is marked; in severe renal impairment, there is a tendency toward equalization of the hourly portions.

DYE TESTS

The kidney can eliminate *via* the urine certain foreign substances that reach the blood after oral, intravenous or intramuscular administration. Many tests have been proposed on this basis including a number of different dyes. In 1912, Rowntree and Geraghty published their phenolsulfonephthalein test which has attained wide popularity among clinicians. The original test, in which the dye was injected intramuscularly, has been modified by Shaw who administered it intravenously. This modified method has been found to closely parallel the urea clearance test. In advanced stages of nephritis the phthalein output approaches zero and may fail to indicate further changes in the renal condition.

1. **Phenolsulfonephthalein Test.**—The sterile dye solution is injected intravenously or intramuscularly and the percentages of the dye excreted at definite intervals are estimated colorimetrically.

1. **Reagents.**—(a) *Dye Solution.*—This is ordinarily obtained in ampules containing about 1.3 cc., each cubic centimeter equivalent to 6 mg. of dye. It may be prepared by diluting 0.6 gm. of the dye and 0.84 cc. of 2 N sodium hydroxide to 100 cc. with 0.75 per cent sodium chloride solution. The monosodium salt is formed, which is irritating when injected.

When the dye is golden-yellow, in alkaline solution it is a bright purplish-red, a color that can be easily compared colorimetrically for quantitative determination.

(b) *Sodium Bicarbonate.*—Weigh out 4 gm. amounts.

(c) *Sodium Hydroxide Solution*.—Ten to 25 per cent in water.

(d) *Alcohol*.—For sterilization of skin at injection site.

(e) *Standard Dye Solution*.—One cubic centimeter of dye solution (6 mg.) is mixed with 800 cc. of distilled water. Add 4 gm. of sodium bicarbonate or sufficient 25 per cent sodium hydroxide solution (several drops) to produce the maximal red color. Dilute to 1000 cc. with water. This is the 100 per cent standard solution. It will keep, if stoppered and protected from light, for three months in non-soluble glass containers. The standard 100 per cent solution can be made up freshly on each test day by using the small amount of dye remaining in the ampule after giving the 1 cc. injection. Since 100 cc. of standard is amply sufficient for the colorimetric comparisons, 0.1 cc. of the dye solution (6 mg. per cc.) is diluted to 50 cc. with water, a drop of 25 per cent sodium hydroxide solution added, and dilution continued to 100 cc. with water.

2. *Procedure*.—(a) *Preparation of Patient*.—The patient is given 300 to 500 cc. (2 glasses) of water to drink, to insure free urinary excretion. The bladder is emptied, by a catheter if necessary, and the urine discarded. Twenty to thirty minutes later 1 cc. of the dye, accurately measured, is injected into a vein at the elbow. Intramuscular administration into the deltoid, gluteal or lumbar muscles may be preferred. The time of the injection is noted.

(b) *Collection of Specimens*.—(1) *After Intravenous Injection*: Specimens of urine are collected, with complete voiding each time, at fifteen, thirty, sixty and possibly one hundred twenty minutes. Each bottle is properly labeled. If several hours are to elapse before the urine is examined, render it acid with phosphoric acid. If more than 25 per cent is recovered in the first fifteen-minute specimen, the function can be considered normal and further specimens need not be taken.

(2) *After Intramuscular Injection*: Specimens are collected at seventy and one hundred thirty minutes from the time of the injection. All the urine is voided into separate receptacles labeled No. 1 and No. 2. With the intramuscular method a single specimen collected at one hundred thirty minutes will give as much information as the divided specimen.

(c) *Estimation of Output*.—Each specimen is placed in a 1000 cc. cylinder or volumetric flask; the amount of each specimen is measured and recorded. If below 40 cc., results are not dependable. Add water to bring each to about 500 cc. Add to each 4 gm. of sodium bicarbonate or 5 cc. of 10 per cent NaOH, or a sufficient number of drops of 25 per cent sodium hydroxide solution to produce a maximal purplish-red color. Add water to the 1000 cc. mark. Mix by inverting several times.

From each diluted and alkalized specimen, filter an amount sufficient for the colorimetric comparisons. In using a colorimeter of the Dubosq type more accurate readings are obtained if the colors of the standard

Since 50 per cent
specimen, the 100 per
cent of distilled water to
make a 50 per cent standard. The colorimeter should be standardized before use in the usual manner. The standard is usually set at 10, when the calculation becomes:

$$\frac{\text{Reading of standard (10)}}{\text{Reading of unknown}} \times 50 \text{ (per cent value of standard used)} = \text{per cent of dye excreted.}$$

The Dunning colorimeter can be used. It consists of thirteen sealed ampules containing standard color solutions of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 80 and 100 per cent. These maintain their accuracy for about one year. There is also an open ampule in which the diluted alkalized specimen is placed; this ampule is placed in the center slot of the three slot comparison box provided; the standards are placed alongside the unknown until a match is made; the percentage is read on the matching standard ampule. If no colorimeter is available, dilutions of the 100 per cent standard in a range between 5 and 50 per cent can be made up in test tubes of uniform size, and the urine specimens in similar sized tubes compared with them.

An alternate method is to fill one 50 cc. graduated cylinder to the mark with the prepared urine specimen; in a second similar cylinder place 10 cc. of a 50 or 25 per cent standard dye solution, depending on the depth of the color of the urine specimen. Add water, a few cubic centimeters at a time, to the standard cylinder until the color matches the urine specimen, when viewed through the side of the cylinder against a white background. Read the level of the total fluid column in the standard cylinder (this value is R in the formula). The calculation is:

$$\frac{10}{R} \times \text{percentage value of standard solution} = \text{percentage of dye excretion}$$

3. Results.—(a) *Normal in Intravenous Method.*—Excretion of the dye begins in three to five minutes. In the table below are given the percentages of dye excreted by the normal kidneys. Quantities below the minimum figures are to be regarded as indicative of impaired renal function.

TABLE 3			
Times, min.	Minimum per cent	Maximum, per cent	Average, per cent
15	25	50	35
30	40	60	50
60	50	75	65
120	55	85	70

(b) *Normal in Intramuscular Method.*—Excretion begins five to eleven minutes after the injection. To allow for this, the specimens are collected at seventy and one hundred thirty minutes. At the end of seventy minutes 40 to 60 per cent of the dye is excreted; at one hundred thirty minutes, a total of 60 to 85 per cent. In actual practice, all values over 50 per cent in the two hour period are considered normal.

4. Interpretation.—In normal individuals the quantity of dye excreted is independent of urine volume, but with severe renal impairment, the output of phthalein varies more or less directly with the urine volume, and can be increased by the liberal use of water.

Mild nephritis gives only a delayed output, resulting in the recovery of less than 25 per cent in the first fifteen minutes, although over 60 per cent may be the total recovery at one hundred twenty minutes. In essential hypertension and nephrosclerosis the rate of excretion may be delayed. In advanced renal disease the excretory rate is diminished as well as the total output at one hundred twenty minutes. The method is positive before the blood shows retention values, the intravenous test closely paralleling the urea clearance figures.

In extra-renal conditions, such as cardiac failure and other conditions causing pre-renal deviation of water, low values are found, the output falling to 50 per cent or less of the normal, while the concentration and clearance tests are normal. In cystitis, pyelonephritis and prostatic obstruction, low percentages are met.

Before employing the test, be sure that there is no urinary obstruction. At sampling time be sure all urine is voided. If all extra-renal causes are excluded, an output of 10 per cent in one hundred twenty minutes indicates severe renal dysfunction with life expectancy a matter of months. When the output falls to 5 per cent or less, the test can no longer be used to follow the progress of the disease; resort must then be had to blood retention studies.

5. The Test in Urological Examinations.—The test may be used to advantage to determine the function of the two kidneys separately. After ureteral catheterization and emptying of the bladder, the dye is injected intravenously. The catheter ends are inserted into test tubes containing a few cubic centimeters of 1 per cent sodium carbonate solution. As the urine drops into these containers the first appearance of a red color is noted. Dye should appear in two to five minutes; occasionally there is a reflex delay to eight minutes. At fifteen minutes each kidney should excrete at least 15 per cent. If one is diseased, less than 15 per cent is excreted by it and the appearance time will be prolonged; the other may compensate by eliminating more than 15 per cent. Both kidneys eliminate 35 to 45 per cent in fifteen minutes, 50 to 60 per cent in the first thirty minutes, and 60 to 80 per cent in one hour. After removing the catheters the bladder should be emptied to see if any dye has leaked by them; such leakage causes decreased values.

6. Test in the Presence of Bile Pigment in the Urine.—Bile pigment if present must be removed. This is done by precipitation as the barium salt, the phthalein excretion being determined on the filtrate.

(a) *Procedure.*—The dye is administered in the usual manner. One specimen of urine is collected at one hundred twenty or one hundred thirty minutes depending upon the route of administration. Divide the specimen into two equal parts, placing each portion in a 1000 cc. volumetric flask. Add to the first flask saturated barium hydroxide solution in an amount equal to the urine plus an excess of 50 cc. Add water to the mark, invert several times and allow the precipitate of bile pigment to settle out for a few minutes. Filter a sufficient amount for colorimetric determination against the 50 per cent standard. If the percentage is above 50, the value is accepted without correction. If the reading is below 50 per cent, the low reading may be due to adsorption of the dye by the precipitate. To obtain per

mine the percentage of dye present in the filtrate. The value obtained on the first flask (A) plus 50 per cent is the expected value of the second flask specimen. If the expected value is actually found, no correction need be applied to (A). If a lower result is obtained some dye is adsorbed and correction of (A) is necessary. The formula used is:

$$\frac{50}{50 - [(A + 50) - B]} \times A = \text{the corrected percentage of dye excreted,}$$

where A is the percentage in the first flask and B that of the second to which dye was added. For example: If the value of the first flask is 30 (A), and the second is 60 (B) instead of the expected 80, then 20 per cent of dye was adsorbed. Substituting in the formula we get:

$$\frac{50}{50 - [(30 + 50) - 60]} \times 30 \text{ or } \frac{50}{50 - 20} \times 30 \text{ or } \frac{1}{2} \times 30 = 50 \text{ per cent.}$$

UREA CLEARANCE TEST

I. Basis of Test.—The ratio $\frac{U \text{ (Concentration of urea in the urine)}}{B \text{ (Concentration of urea in the blood)}}$ was utilized in 1904 by Grehant as an index of the functional capacity of the kidneys. Ambard and Weill in 1912, utilized the same ratio but took into consideration the two variable factors of urea output and urine volume. Their complex formula was simplified in 1915 by McLean. In 1916 Addis and Watanabe suggested the ratio between the rate of urea excretion and the blood urea concentration as a more accurate index of renal efficiency. In 1928 Moeller, McIntosh and Van Slyke introduced the urea clearance test as a measure of renal function. The test has been improved by further study so that now it is easily and accurately performed and gives highly accurate results.

The urea clearance test serves as an indicator of glomerular function; fall in the clearance is a function, though not a simple direct proportion, of the number of glomeruli destroyed. The concentration tests are very sensitive to slight degrees of renal damage but they fail to show differences between moderate and severe renal impairment. If the concentration test is normal it is generally not necessary to perform further studies. But if it yields a urine of low specific gravity, it is necessary to determine the urea clearance also to ascertain whether the decreased excreting power is serious. Excretory power and clinical condition follow the clearance—not the specific gravity.

The test is based upon the quantity of blood cleared of urea per minute while passing through the kidneys. Although the quantity of urea eliminated varies with the protein intake, the percentage eliminated is quite constant under normal conditions. In determining the urea clearance three factors are considered: (1) The concentration of urea in the blood, (2) the concentration of urea in the urine and (3) the urine volume per unit of time.

In the normal adult passing a urine volume of 2 cc. or more per minute ("Augmentation limit") the urea excretion is at a maximum value. This maximum clearance, C_m , ranges from 64 to 99 cc., averaging 75 cc., of blood cleared of urea per minute. When the output is less than 2 cc. per minute, the volume of blood cleared of urea each minute varies from 40 to 60 cc., with a mean value of 54 cc. This is designated as the standard clearance, C_s .

II. Calculations.—The clearance values can be calculated from formulas involving the use of blood and urine urea contents and urine volume as follows:

$$\text{Maximal clearance } (C_m) = \frac{U \times V}{B}$$

$$\text{Standard clearance } (C_s) = \frac{U \times \sqrt{V}}{B}$$

U = urea in mg. per 100 cc. of urine; B = urea in mg. per 100 cc. of blood; V = volume of urine excreted per minute.

Results may be expressed either as cubic centimeters of blood cleared of urea per minute or, preferably, as percentages of the normal clearance. To convert the actual clearance to percentage of normal the values obtained in the above formulæ may be multiplied by 100 and then divided by the normal clearance values, 75 for the maximum and 54 for the standard clearance.

The formulæ then become:

$$\text{Percentage of } C_m = \frac{100 UV}{75B} \text{ or } \frac{1.33 UV}{B}$$

$$\text{Percentage of } C_s = \frac{100 U\sqrt{V}}{54B} \text{ or } \frac{1.85 U\sqrt{V}}{B}$$

In children and in adults who vary widely from the normal surface area of 1.73 square meters, a correction factor must be employed. In individuals between 62 and 71 inches in height no correction is required as the error involved is less than 5 per cent. In others, the urine volume must be multiplied by the correction factor, the formulæ to use becoming:

$$\frac{\text{Body surface area in square meters of a normal adult}}{\text{Body surface area in square meters of patient}} = \frac{1.73}{A}$$

or

$$C_m = \frac{U}{B} \times \frac{V \times 1.73}{A}$$

$$C_s = \frac{U}{B} \times \sqrt{\frac{V \times 1.73}{A}}$$

The surface area of patients can be obtained from tables used in basal metabolism calculations.

III. Factors Influencing the Clearance.—It has been shown that in normal individuals the urea clearance varies with the renal blood flow, which changes spontaneously from minute to minute. Consequently, variation of the clearance within certain limits is a natural occurrence. It is an evidence of normal functional elasticity of the kidney. The usual range is from 75 to 130 per cent of the average normal, variations of this nature are to be expected.

Caffeine, milk and small doses of epinephrine increase maximal clearance; pituitrin and large doses of epinephrine decrease it. The clearance is depressed during the first hour after rising, and begins to slowly and regularly rise from before breakfast to noon; there is a definite decrease after lunch, with a subsequent rise in the late afternoon and evening. Exercise produces a marked decrease in the clearance. In planning the procedure for the test, these factors are considered.

IV. Procedure.—There is no preliminary preparation of the patient.

Except in advanced cases of nephritis, it makes but little difference whether the patient lies down or walks about during the test. Vigorous exercise should be avoided. In advanced cases of nephritis when the clearance has fallen to below 50 per cent patients should rest in a reclining

ing position. The test is best performed in the morning between 9 and 12 o'clock when excretion fluctuations are minimal.

A fairly free flow of urine should be promoted during the test to avoid error caused by retention of urine in the urinary passages and bladder. For this reason it is customary to give two glasses of water, one at the beginning of the test and one an hour later. Two specimens of urine are collected at approximately hourly intervals, with blood drawn at the midpoint of the test. Two clearances are thus determined.

After drinking the first glass of water, the patient empties his bladder completely, discarding the specimen. The time is accurately recorded or a stopwatch started. One hour later the patient voids completely, the specimen is saved and the time accurately noted. At the end of another hour the bladder is again completely emptied, this urine also being saved. It is not necessary that the urine be passed at exactly hourly intervals. It must be passed at least 50 cc. of urine are

be obtained. Provided the time is accurately known, the volume of urine per minute or per hour may be easily calculated.

Blood is drawn near the midpoint of the test; it may be taken just before or after the second voiding. The exact time is unimportant as the fluctuation of the blood urea is but slight over such periods of time.

After the specimens have been collected, the urine volume is determined within 1 per cent. Blood and urine urea concentrations are then determined and the clearance values calculated by use of the formulæ given above. For methods of determining the urea nitrogen and calculating the clearance see page 201.

V. Interpretation.—When properly performed the test has a high degree of accuracy. Low results may be the result of incomplete voiding. Dehydration as the result of diarrhea or vomiting, especially in children, gives low clearance values although renal pathology is absent.

The concentration tests will usually detect impairment before the urea clearance. When the clearance shows damage to the kidneys, the concentration of the urine is apt to be fixed and thus can show no further changes. The clearance will enable the physician to follow the continued progress of the patient. In acute nephritis, if healing is to occur, the clearance will usually return to normal within four months after the onset, while the concentration may be reduced for many months longer.

The urea clearance and the intravenous phenolsulfonephthalein test will often closely parallel one another; the intramuscular dye test will often be within normal limits until later in the disease. In obstruction and urinary retention the phthalein test may be low compared with the clearance, since only a fraction of the phthalein is likely to be voided in the two hours. The urea clearance may vary markedly in normal individuals but in advancing renal impairment it tends to become fixed.

Clearance values will be markedly reduced before there is retention of nitrogen in the blood. Blood studies will, however, show further progress when the clearance has been reduced to about 5 per cent, at which point it tends to become fixed. The elimination or the deposition of excreta fluid does not affect the test, so it is to be preferred in such cases.

The maximum clearance is normally about 40 per cent greater than the

standard clearance. Values of 75 per cent of normal are considered as indicating unimpaired renal function; those between 75 and 50 per cent doubtful; and those below 50 are always indicative of renal inefficiency, if cardiac failure can be ruled out. The pathological decrease in the clearance values is due either to decrease in the per minute volume flow of blood through the kidneys or diminution in the proportion of urea removed from the blood in its passage through these organs. In early diffuse acute nephritis with red blood cells and casts in the urine, the clearance may be normal, but in the majority of such cases the clearance is below 50 per cent. If there is no definite rise toward normal within four months, the condition progresses to a chronic or terminal stage.

When the clearance falls below 10 per cent, premonitory symptoms of impending uremia appear; if it falls to 5 per cent, uremia may be considered as established. While death may occur quickly in such cases, in others it may be delayed for some months. In arteriosclerotic Bright's disease and

In chronic glomerulonephritis the clearance may be normal during the latent stage; with activity of the disease process the values are less than 60 and usually under 40 per cent. Stationary periods in the clearance values are common and may last for months, then the downward course is resumed. When the clearance falls below 20 per cent in these cases, the life expectancy is, at the most, about two years, the majority succumbing within a year. In true lipoid nephrosis the urea clearance is normal. In the nephrotic stage of glomerulonephritis the clearance may be normal or slightly lowered. With the passage of time the clearance drops and the picture becomes similar to that of the usual glomerulonephritis. In the later months of pregnancy the clearance is low. Occasionally a clearance below 50 is due to cardiac decompensation; in such cases the urine tends to have a high specific gravity.

BLOOD NITROGEN RETENTION STUDIES

Blood chemistry determinations for detecting the accumulation of non-protein nitrogen, urea nitrogen, uric acid and creatinine are more important from the standpoint of prognosis and the management of renal hypofunction than from that of diagnosis. There is evidence of retention only after the reserve power of the kidneys has been exhausted and other tests of renal function are positive. Proper restriction of food intake or a compensatory polyuria may suffice to relieve the strain on the renal excretory powers, so that even in advanced renal disease blood determinations may be practically normal. Blood chemistry studies become important in advanced decompensated renal impairment when other tests may fail to show further progress.

The concentration of non-protein nitrogenous substances in the urine far exceeds their content in the blood. Their elimination is largely dependent on the available water. Pre-renal deviation of water, as in edema or dehydration, or increased protein catabolism, as in high protein intake, pyrexia, and toxic destruction of tissue protein, tend to produce nitrogen retention by increasing the excretory load on the kidneys, especially if

decreased concentrating power is already present. An evaluation of the renal as well as the extra-renal factors present is necessary. Usually when it is due to defective renal function the specific gravity of the urine is low. Nitrogen retention has less significance on a high protein intake than when present on a low protein nephritic diet.

I. Urea Nitrogen Retention.—Ordinarily it should not be necessary to do a complete blood chemistry examination to determine the degree of nitrogen retention. It is more advantageous to determine the urea nitrogen rather than the non-protein nitrogen; ordinarily creatinine need not be determined unless the urea nitrogen is over 30 mg. per 100 cc.

1. Normal Values.—The normal concentration of urea nitrogen in the blood is 10 to 15 mg. per 100 cc. averaging about 12. The urea nitrogen is approximately half the non-protein nitrogen, but in retention it may constitute 80 to 90 per cent.

2. Values in Renal Disorders.—In the late stages of chronic nephrosis normal values are to be expected. In acute nephrosis or anuria in the necrotizing nephroses, such as in the acute stages of the disease, values may rise up to 300 mg. per 100 cc.

Values in the range of 100 to 200 mg. per 100 cc. indicate a moderate degree of retention, and values above 200 mg. per 100 cc. indicate a severe degree of retention. Persistence of high values indicates the establishment of chronicity.

High values in chronic nephritis are of more serious import than in acute or acute exacerbation types. In chronic nephritis a concentration of 50 mg. per 100 cc. is serious, but prolonged survival is possible; values of 50 to 100 mg. prognosticate death usually within a year; 100 to 150, death within a few weeks or months; over 150, death is a matter of a few weeks or even days.

In advanced renal sclerosis or in urinary obstruction, increased values are constant but extra-renal factors must be ruled out. In the surgical kidney increased values are frequently found. Patients with retention due to obstruction may have values over 100 mg. per 100 cc. and yet not appear ill. If casts are present in the urine a coexisting nephritis is suggested. Patients with values over 35 mg. are poor operative risks.

In essential hypertension, as long as there is neither renal nor cardiac failure, the urea nitrogen is normal. With the onset of renal insufficiency and at times with cardiac failure, the blood urea nitrogen rises. This rise may be marked in cases of severe oliguria or temporary anuria accompanying a complicating acute coronary closure; this quickly clears if the heart recovers.

II. Non-protein Nitrogen Retention.—The total non-protein nitrogen in the blood is determined by the extent of the renal lesion, on the existence of extra-renal factors and on the extent of pre-renal deviation of water.

1. Normal Values.—These vary between 25 and 35 mg. per 100 cc. of blood.

2. Values in Renal Pathology.—In acute nephritis there is a variation from slight retention (non-protein nitrogen 40) to extremely high figures (non-protein nitrogen 300). The severity of the renal impairment may be discounted somewhat when the urine concentration is high. The marked

retention is often due to extra-renal factors, especially the pre-renal deviation of water that accompanies excessive vomiting, edema or fever. In acute nephritis the subsidence of the acute process is accompanied by a recession of the blood nitrogen values toward normal. Nitrogen retention with a low specific gravity urine is more significant of renal impairment than a similar degree of retention with a high gravity urine.

In chronic glomerulonephritis it is the renal impairment that causes the nitrogen retention. When the retention becomes apparent it usually progresses with varying rapidity toward the final stages of the disease. The urea nitrogen constitutes a large part of the total non-protein nitrogen ~~increase~~ the creatinine remaining for a considerable time within normal

than renal insufficiency. In destructive parenchymatous lesions of the kidneys, such as polycystic disease or tuberculosis, the presence of nitrogen retention indicates that over two-thirds of the total kidney tissue is involved. Single non-protein nitrogen determinations are of little value. It is the persistence rather than the degree of the retention that is important. Increased retention persisting after proper therapeutic or surgical measures means a bad prognosis. In urinary obstruction the degree of retention may be as high as 400 mg., yet on the relief of the obstruction, the nitrogen level may be restored to normal. In chronic passive congestion, urea nitrogen and non-protein nitrogen show little change, but the phthalein output is low.

III. Creatinine.—The normal limits of creatinine in the blood of 1 to 2 mg. per 100 cc., are relatively fixed. As it is readily excreted, retention occurs only in serious renal impairment. The creatinine value is of considerable importance to the clinician from a prognostic standpoint; it increases too late in the course of renal impairment to be of use in diagnosis. In chronic nephritis, with uremia, values up to 35 mg. per 100 cc. of blood may be found; in uremia a value over 5 mg. indicates a fatal termination. In acute nephritis and in acute exacerbations of chronic nephritis, extremely high concentrations may occur; yet with subsidence of the acute process the creatinine returns to normal. A maintained elevation of only a point or two above the normal for the patient under observation is serious, except in acute nephritis or in cases of urinary obstruction. In the urological patient the retention of urea tends to be higher than in nephritic cases having the same blood creatinine concentration.

IV. Uric Acid.—As elevations of the uric acid concentration occur in other conditions, it is not a very satisfactory indicator of renal hypofunction. The normal limits are 1.5 to 3.5 mg. per 100 cc. of blood. The level is not constant, varying from hour to hour. Uric acid is eliminated with more difficulty than either urea or creatinine. Its value is usually increased in both acute and chronic nephritis when there is nitrogen retention. Its retention may precede that of urea or creatinine. Values of 4 to 25 mg. per 100 cc. may be present; over 30 is rare. In contradistinction to nephritis, in gout the value rarely exceeds 9 mg. per 100 cc. In hypertension there is a slight increase which does not appear to portend renal failure.

MISCELLANEOUS

1. Congo-red Test for Amyloidosis.—Amyloid absorbs Congo-red more than normal tissue. The kidneys freely excrete the dye when injected intravenously, but much less is recovered in the urine in one hour if amyloidosis is present.

1. Reagent.—*Congo-red Solution.*—Dissolve 300 mg. of Congo-red of the highest purity in 20 cc. of freshly distilled water making a 1.5 per cent solution. Filter and sterilize in the autoclave or by boiling. The solution is to be used immediately after opening the bottle or ampule and any remaining is discarded.

2. Procedure.—The solution of Congo-red is administered intravenously, 0.25 cc. being given for each kilogram of body weight, with a maximum dosage of 18 cc. The average adult will receive 15 cc. The proper dose is taken up in a 20-cc. syringe, and injected into an antecubital vein. Four minutes later, 10 cc. of blood are removed from a vein in the opposite arm, using a fresh syringe and needle. The blood is placed in an oxalated centrifuge tube, corked, inverted several times to mix, and labeled. One hour after the dye injection, a second blood specimen is collected in a second oxalated centrifuge tube. Both specimens are centrifugalized for twenty minutes and the plasma pipetted off. The colors of the two specimens are compared in a colorimeter, the four minute plasma being considered as the 100 per cent standard. It is placed in the left hand cup of the colorimeter and set at 10 mm. The sixty minute plasma is matched against it. The formula for the calculation is:

$$\frac{10 \text{ (reading of standard)} \times 100}{\text{Reading of one-hour specimen}} = \text{percentage dye in one hour.}$$

One hundred minus the percentage of dye in the one-hour specimen equals the percentage of the dye excreted by the kidneys or taken up by the tissues. The urine is collected at the same time the one-hour blood sample is obtained. A deep red color indicates renal excretion and nephrosis; slight pink tints are disregarded.

3. Results.—Normally less than 30 to 40 per cent of the dye disappears from the blood in one hour. In amyloid disease from 40 to 100 per cent disappears from the blood in that period. In chronic nephrosis the dye disappears more quickly than normally, values of 40 to 60 per cent being found, whereas in amyloid disease the value is usually over 60 per cent. Abnormally rapid disappearance of the dye occurs in amyloid nephrosis, chronic nephrosis and the nephrotic type of glomerulonephritis. In nephrosis there is also much dye in the urine. Smaller amounts are found in the urine in glomerulonephritis with edema. If there is a little or no dye in the urine, with disappearance from the blood, amyloidosis is the cause.

CHAPTER III

HORMONAL TESTS FOR PREGNANCY

By CLYDE J. GENTZKOW and HOWARD A. VAN AUKEN

RARELY does the clinician meet with problems in differential diagnosis which are more confusing than those in which the possibility of early pregnancy must be considered. Vaginal bleeding is one of the most frequent symptoms of gynecological pathology and very often necessitates consideration of threatened abortion as a possible diagnosis.

During the past decade and a half certain hormonal tests for early pregnancy have been developed. With experience a high degree of accuracy has been obtained and at the present time, these tests, carefully done, give conclusive evidence of the presence or absence of pregnancy very early indeed.

Characteristic of the Aschheim-Zondek test and of the various modifications is the fact that the reaction of the test animal depends on the presence of certain pregnancy hormones in the blood and urine of the subject. For this reason a brief review of the origin and nature of these hormones is indicated.

It had long been believed that the pituitary was linked up in some way with the ovaries but it required the work of Zondek and Aschheim, Smith and others to establish this relationship. The anterior pituitary controls their activity through the action of two hormones known by a variety of names among which gonadotropic sex hormones and prolan are the most common. One of these, often called the "follicle-stimulating hormone," (F.S.H.) stimulates the development of the ovarian follicles with the resultant production of corpora hemorrhagica.

The ovarian follicle during the process of maturation produces gradually increasing amounts of estrogenic hormone known commonly and sold commercially as estrin, theelin, folliculin, amniotin, etc. Occurring in many different but related forms, the generic term of estrogen has been applied to it because of its ability to produce estrus in castrated animals.

In this discussion only its chief and governing the rhythmic

At approximately the height of maturation of the ovarian follicle the second of the anterior pituitary gonadotropic hormones begins to function. This hormone produces luteinization of the follicle after ovulation has occurred, resulting in the formation of the corpus luteum. Once luteinization begins the follicle then starts secreting its second hormone, progesterone. This hormone has two important functions. One is to produce secretory changes in the already built up endometrium, changes necessary before implantation can take place. The other function is inhibition

hormone found in the blood and excreted in large quantities in the urine in pregnancy, was in reality that produced by the anterior pituitary. The increased

amount found in the urine was believed to be due to increased activity of the gland during gestation.

It has been proved more recently, however, that the hormone of pregnancy urine, anterior pituitary-like in its actions, is in reality secreted by the trophoblast of the fertilized ovum. It is, therefore, properly known as the *trophoblastic* or *chorionic gonadotrophic* hormone, the name given it by Novak, and it is upon the presence of this hormone that most tests for pregnancy are based. This chorionic gonadotropin is also dual in character, having both follicle-stimulating and luteinizing components.

In addition to producing chorionic gonadotropin, the placenta elaborates large amounts of estrogenic hormone and also of progesterone.

Whether the chorionic gonadotropic hormone and the estrogenic hormone are each single entities or are composed of several hormones, each with different though similar functions is a moot question. It is definitely known, however, that each appears in the urine as several different but chemically related compounds.

METHODS

I. The Aschheim-Zondek Test.—1. **Materials.**—(a) *Urine Specimen.*—Collect a fresh morning specimen in a *clean* container. Sterility is not necessary. At least 25 cc. are required. It is well to restrict fluids the previous night in order to secure a more concentrated specimen. Chill immediately and keep refrigerated until used. If chemical preservation is necessary, add 1 drop of tricresol per 25 cc. of urine. All medication should be stopped since some drugs, such as ergot, quinine, arsenic, barbiturates, morphine, codeine and aspirin, may be excreted in the urine and may kill the test animals.

(b) *Animals.*—Five immature female white mice, twenty-one to thirty days old, weighing 6 to 8 gm.

2. **Procedure.**—Warm the urine to about 40° C., not more. If the specimen is cloudy, filter it; if alkaline, acidify faintly with dilute acetic acid. Use a tuberculin syringe and a 24-gauge needle 1½ inches long. Inject 0.4 cc. of the urine subcutaneously into each of 5 mice three times a day for two days, making a total of six doses. Kill the animals ninety-six to one hundred hours after the first injection using ether or illuminating gas. Autopsy the animals carefully in order to prevent escape of blood into the abdominal cavity, for such leakage may mask the ovaries and tubes.

3. **Results.**—In most cases the changes are visible with the naked eye. When necessary a hand lens may be employed.

(a) *Negative.*—The ovaries and tubes are white and small, the ovary rarely being larger than 3 mm.

(b) *Positive.*—Aschheim describes three types of reaction:

Reaction I—consists of follicular ripening with hyperemia and swelling of the follicle with secondary estrus.

Reaction II—follicular hemorrhage with the formation of corpora hemorrhagica.

Reaction III—follicular luteinization with formation of corpora lutea atretica.

Only reactions II and III may be considered as evidence of pregnancy. The ovaries will be enlarged and hyperemic with bright red points 0.5 to

1 mm. in diameter projecting above the surface. The presence of a single corpus may be considered a positive reaction. Corpora lutea are indicated by grayish yellow spots.

Occasionally it may be necessary to examine the ovaries microscopically. This can be done by pressing the ovaries between two glass slides held together by a rubber band.

In addition to the above the effects of the ovarian hormone may be visible, as evidenced by enlargement and hyperemia of the uterus, distention, enlargement and hyperemia of the tubes, and premature opening of the vagina.

1. **Detoxification of Urine.**—Some urines may be toxic to the animals. Many, but not all may be detoxified by the method of Zondek. Filter 30 cc. of the fresh urine and add 90 cc. of ether. Shake vigorously for five minutes in a separatory funnel and allow to layer. Draw off the urine into a beaker and allow to stand in the open air until the residual ether evaporates. Dissolve 0.9 gm. of glucose in this ether-extracted urine, and keep the specimen in a refrigerator until used.

II. **The Friedman Test.**—The use of mice is open to certain objections: (1) a sufficient constant supply of immature animals is hard to maintain, (2) results may be difficult to read, (3) the test requires ninety-six hours for completion, (4) the method with its multiple injections into multiple animals is tedious.

1. **Materials.**—(a) *Urine Specimen.*—The same as that described above for the Aschheim-Zondek test.

(b) *Animals.*—One rabbit is used for each test. The rabbit should be a healthy female, unmated, non-pregnant, over three months old, 4 pounds or more in weight. Females should be kept separated from males for three weeks to one month before use, since the gestation period is about thirty days. The test animal should be isolated for seven to ten days to obviate any mechanical stimulation of sexual organs that might produce confusing ovulation.

2. **Procedure.**—The urine is warmed, filtered and acidified, as above, if necessary. Using a 10-cc syringe and 20- to 22-gauge needle, depending upon the size of the ear vein of the rabbit, slowly inject 10 cc. of urine into the vein. Stroking the ear, applying warmth or xylene to it or clamping near the base with a paper clip will bring out the vein. The animal is anesthetized and the ovaries examined through an abdominal incision forty-eight hours after injecting the urine.

3. **Results.**—(a) *Negative.*—The ovary is cylindrical, white or pale pink and studded with small, clear, unripe follicles. The uterus is not injected.

(b) *Positive.*—The ovaries are studded with 1 to 6 or more corpora hemorrhagica, appearing as dark red blood points, projecting above the surface. Corpora lutea may be present as yellow spots. Rose spots in large clear follicles are suspicious, requiring repetition of the test. The uterus and tubes are injected and coiling is increased. Luteinization begins in the corpora hemorrhagica after forty-eight hours.

A flat, small opaque and smooth ovary is suggestive of an immature animal not sufficiently developed to respond to any stimulation. Occasionally a false positive is encountered in which the spontaneous formation of corpora hemorrhagica has occurred. These corpora appear dull red and rounded, with the uterus usually small and pale.

NOTE.—The animals may be re-used provided they are opened under anesthesia, observing the usual sterile technic. Negative animals can be used again immediately. Rabbits showing a positive result can be used after being isolated for ten to fourteen days. The abdominal wound should be closed in two layers.

III. Frog Test (Bellerby, 1934).—The use of the South African clawed frog, *Xenopus laevis*, for the diagnosis of early pregnancy has many advantages. The test is based upon the fact that the mature female frog carries eggs throughout the year which are extruded only after mating or following the stimulation of the hormones peculiar to pregnancy. By keeping the frogs isolated from the male, they become an ideal test animal. The result of the test may be determined in four to eighteen hours, no operation is necessary, there is but one injection to be given and the inexpensive animals can be used over and over again. The test, if properly performed, is at least as accurate as either of the above described tests.

1. Materials.—(a) Urine Specimen.—Whole fresh urine may be used but a concentrate is more satisfactory. Acidify 80 cc. of fresh morning urine faintly with acetic acid. Add 160 cc. of acetone, mix and let stand for fifteen minutes. Proteins and the hormone precipitate out. Centrifugalize, decant the supernatant fluid and wash the precipitate twice using 20 cc. of ether each time. Allow to dry, stir up with 2 cc. of water and centrifugalize again. The hormone goes into solution but the protein does not. Just prior to injection pour off the 2 cc. of solution, adjust the pH to 5.5 using sulfosalicylic acid with nitrazine paper as the indicator. One cubic centimeter of this 10 times concentrate is injected into a frog, the other being retained for recheck if desired, or a second animal may be injected immediately.

(b) Animals.—One or two female frogs (*Xenopus laevis*). The frogs may be kept in large tanks, covered with water to a depth of 3 inches. Two tanks of about 10 gallons capacity will be required for each 10 animals. One tank is reserved as a rest tank for those animals which have been used; recuperation requires about four weeks. In the other are kept the animals ready for immediate use. The frogs are fed small strips of beef heart, liver and garden worms (if available) twice a week.

2. Procedure.—Draw 1 cc. of the urine concentrate up into a small syringe. Exercising care, thrust the needle through the skin of the frog, then direct the needle throughout its course, the needle should be seen clearly just beneath the skin. A firm gentle thrust carries the needle through the connective tissue into the dorsal lymph sac. Great care is necessary to keep the needle from entering the left lung and killing the animal. Inject the urine concentrate into the sac and withdraw the needle.

Place the animal in a 2 gallon jar, filled to a depth of 3 inches with water. A wire mesh screen should be placed about an inch above the bottom of the jar to prevent the animal from eating its own ova. The temperature of the water in all tanks should be the same, and should be kept fairly constant. Keep the tanks in a bright, airy place. Examine the jars periodically, beginning four hours after the injection and continuing for eighteen hours. After each test turn out any eggs which may adhere to the wire.

3. **Results.**—(a) *Negative*.—No ova are deposited over a period of eighteen hours.

(b) *Positive*.—The appearance of ova which are easily observed macroscopically is a positive result. Usually hundreds of ova will be deposited. At the conclusion of the observation the animal is placed in the rest tank for recuperation.

IV. Estrin Test for Pregnancy.—This test, based upon the demonstration of estrin in the urine of the pregnant woman, depends upon the changes induced in the lower genital tract of the castrated female mouse.

1. **Materials.**—(a) *Urine Specimen*.—As described for the Aschheim-Zondek test.

(b) *Animals*.—Castrated, adult, female, white mice, 3 to 5.

2. **Procedure.**—Prepare the urine as above. Inject 15 cc. subcutaneously into each of the mice in 6 divided doses over a period of two days. At the end of four days after the first injection vaginal smears are prepared and examined microscopically.

3. **Results.**—(a) *Negative*.—The smear consists primarily of leucocytes and mucus.

(b) *Positive*.—Non-nucleated epithelial cells predominate, showing cornification of the vaginal mucosa; leucocytes and mucus are absent. Mild reactions with some non-nucleated cells present but also showing a number of leucocytes and some mucus cannot be considered positive.

V. Interpretation of Results.—These tests are approximately 98 per cent accurate in diagnosing early pregnancy. They become positive within four to five

tion was indefinite. Should the tests become negative after a positive reaction, pregnancy has been made, it is certain evidence of fetal death; otherwise they continue positive until five to seven days after parturition. In ectopic gestation, a positive reaction is obtained in only about 50 per cent of the cases. The estrin test is not positive as early as those tests based on the presence of chorionic gonadotropin in the urine. Fewer false positives are obtained, however.

The Aschheim-Zondek, Friedman and frog tests may give false positives in such conditions as the early menopause, hyperthyroidism, ovarian cysts, endometrial hyperplasia or uterine carcinoma. Primary ovarian failure, where pituitary compensation has taken place, may also give a positive reaction. Performance of the estrin test may serve as a check on the diagnosis in these cases.

VI. Tumor Diagnosis.—**Quantitative Aschheim-Zondek Test.**—As long as viable placental tissue is present these tests are positive. Thus, in

disorders as in pregnancy.

A. In Females.—A quantitative test is carried out diluting the urine 1:10, 1:50, 1:100 and 1:1000. Inject 0.5 cc. of these diluted urines into each of 5 mice as for the Aschheim-Zondek test. If the 1:10 dilution gives a positive result not less than 3330 mouse units of hormone per liter of urine are indicated. Correspondingly higher concentrations are present if the higher dilutions are positive. While the hormone is usually present

in increased amount in chorionepithelioma, a negative result does not necessarily exclude this condition.

B. In Males.—Chorionic gonadotropin is also found in the urine of males who have teratoma of the testis. It can also be found in the fluid of hydroceles that may accompany these tumors, or in extracts of the tumor tissue. In many of these tumors the amount of hormone present is below 2000 units per liter, which is the smallest amount that produces visible macroscopic changes in test animals. The usual tests, employing the urine as passed, would in such cases give negative results. In order to demonstrate macroscopically the presence of the hormone in urine from the less malignant types of teratoma testis, some concentration method must be used.

1. Concentration Method.—(a) *Preparation of Concentrate.*—(1) The fresh morning specimen of urine is filtered if cloudy, and if alkaline is rendered faintly acid to litmus paper with a few drops of weak acetic acid.

(2) Add 100 cc. of 95 per cent alcohol to 20 cc. of this urine and mix by inverting several times. The mixture is allowed to stand overnight, the hormone separating out in the precipitate.

HOUSE	10M	5PM	9AM	10PM	5PM		TOTAL HORMONE PER LITER	EQUVALENT IN MOUSE REACTION 1-12-13	PER LITER
							1-12-13	1-12-13	1-12-13
1	01	01	01	01	01	OF FRESH URINE	05	2000	10000
2	02	02	02	02	02		10	1000	5000
3	04	04	04	04	04		20	500	2500
4	01	01	01	01	01		25	400	2000
5	02	02	02	02	02	OF EXTRACT	30	200	1000
6	04	04	04	04	04		100	100	500

FIG. 4 — (Ferguson.)

(3) The following morning the supernatant fluid is removed, leaving about 20 cc. over the precipitate. This is centrifugalized for five minutes at 2000 r.p.m. The remaining supernatant is poured off.

(4) Add 30 cc. of ether to the precipitate and mix by stirring with a glass rod for ten minutes. Again centrifugalize for five minutes and pour off the ether.

(5) Distribute the precipitate around the bottom of the tube, using a glass rod, and allow it to dry. Add 1 cc. of distilled water; mix and allow to stand overnight.

(6) Centrifugalize this mixture the following morning. The water now contains the hormone; it is pipeted off and kept in the refrigerator until used. This extract is a 5X concentrate of the fresh urine. Stronger extracts may be made by modifying the method.

(b) *Animal Used.*—As in the Aschheim-Zondek test, immature female mice weighing 5 to 8 gm. and twenty to thirty days old are used. In the test 6 of these mice are employed.

(c) *Method and Dose.*—Subcutaneous injection is employed as in the Aschheim-Zondek test. Give each of 3 mice 5 doses of 0.1, 0.2 and 0.4 cc.

respectively of fresh urine and to 3 others similar amounts of the 5X extract as shown in Figure 4. One hundred hours from the time of the first injection the mice are killed with illuminating gas and the ovaries examined. The readings are made both macro- and microscopically, and judged according to reactions I, II and III as described above.

(d) *Results and Interpretations* (According to Ferguson).—Usually male urine contains no chorionic gonadotropin, so the appearance of reaction I has here a specific significance. It appears in urine in cases of teratoma testis as early as two or three months after the known onset of the disease. The amount present in the urine, excluding the chorioneplitheliomas, appears to be in direct proportion to the embryonal character of the tumor, the highest values being found in the embryonal adenocarcinomas, the lowest in the teratomas of adult type. The amount excreted also depends on the mass of viable tumor tissue present; the destruction of the tumor tissue by radiation will be reflected by a decrease in the amount of hormone excreted in the urine.

Negative results do not necessarily exclude the presence of a teratoma testis.

As in pregnancy, the test is positive before clinical evidence appears; the test detects and anticipates metastatic spread or recurrence, when clinical signs are still absent.

The quantitative determination of the hormone output in the teratomas of the testes permits their grading and typing, decrease of malignancy being accompanied by lessened hormone output.

Grade 1 is the chorioneplithelioma of the testis with an output which may exceed 50,000 M.U. per liter, and gives reactions I, II and III in all test mice.

Grade 2 is the embryonal adenocarcinoma, with an output of 10,000 to 40,000 M.U., with reactions I, II and III in all test mice.

Grade 3 is the embryonal carcinoma with lymphoid stroma, giving an output of 2000 to 10,000 M.U., with reactions I and II in all test animals, reaction III appearing only in those animals given the 5X extract.

Grade 4 is the seminoma, with an average output of 1000 M.U., and a range between 400 and 1000 M.U. with reactions similar to Grade 3.

Grade 5 is the teratoma of adult type, giving 50 to 500 units. The mice injected with fresh urine are usually negative, and reaction III does not occur even in mice injected with the 5X extract.

CHAPTER IV

GASTRIC AND DUODENAL FLUIDS

By CLEON J. GENTZKOW and HOWARD A. VAN AUKEN

GASTRIC FLUID

Introduction.—The gastric juice itself is a thin, light colored fluid of complex composition. It is normally acid in reaction, contains about 0.5 per cent of solids and consists of water, hydrochloric acid, sodium chloride, potassium chloride, phosphates, mucin, and the enzymes pepsin, rennin and gastric lipase. It is well to remember, however, that the gastric contents are not only supplied by the activity of the gastric glands but also include a variety of materials swallowed and others regurgitated from the small intestine. Pus, blood, bacteria and tissue fragments from the nose, mouth, pharynx and esophagus may reach the stomach; similar materials from the duodenum and gall bladder may be regurgitated through the pylorus and with the reversal of peristalsis, the fecal contents of the jejunum and ileum may likewise gain entrance.

The exact mode of secretion of the hydrochloric acid of the gastric juice has not been definitely established, although it is known to be produced in the glands of the pyloric third of the stomach. Its concentration may reach 0.4 to 0.5 per cent or pH 1.05, but this maximum is reduced to about 0.2 per cent by the proteins of the food, and by regurgitation from the duodenum. The hydrochloric acid combines loosely with the proteins forming acid metaprotein in preparation for digestion; this acid is designated as "combined HCl." When all of the protein has been thus combined the additional acid secreted remains as "free HCl." Certain acid salts and organic acids may also be present in small amount, adding to the total acidity.

The enzymes pepsin and rennin are also secreted by the glands in the pyloric third of the stomach as the zymogens, pepsinogen and renninogen. Both zymogens are activated by the hydrochloric acid.

There is at all times a slight continuous flow of gastric juice amounting to from a few to 60 cc. per hour. This secretion may be increased greatly in response to many different stimuli. On awakening some individuals increase the quantity secreted; others require the stimulation of the sight or odor of food, the so-called psychic or appetite secretion. The gastric phase of flow is initiated by the passage of food into the stomach, apparently due to the food itself, its breakdown products, distention of the stomach or to a hormone, gastrin, which seems to be identical with histamine. Gastrin acts directly on the acid secreting cells by way of the blood stream. A third phase of secretion, the intestinal, is brought about by the action of small intestine.

The amount of the gastric juice may vary markedly with the food ingested. Meats and high protein foods remain longest (three to four hours). Foods low in protein as fruits and vegetables give rise to low acidities and leave

the stomach soon (one and one-half to two hours). Fats in general have an inhibitory effect on secretion and motility.

Disease conditions may also profoundly alter the character and amount of the gastric juice. Disorders of the stomach may lead to hyper- or hypo-secretion; the same is true of changes in the duodenum. Pathologic conditions in the liver, gall bladder or pancreas may be reflected in the gastric juice. Blood disorders, notably the primary anemias, regularly cause complete suppression of free HCl. It must not be supposed that every change in the gastric juice is caused by disease, however. Hypochlorhydria is a normal finding in some individuals, especially in the older age groups. In some atrophic lesions of the gastric mucosa, there may be persistent suppression of the secretion of hydrochloric acid and of the ferments; this is designated achylia gastrica.

As one of the factors considered in gastric analysis is the amount of contents recovered, the factors concerned in the propulsive movement of the stomach should be kept in mind. Gastric evacuation occurs when the intragastric pressure near the pylorus exceeds the resistance of the sphincter. Different foods are discharged into the duodenum at varying rates, depending on the amount of hydrochloric acid. It

lates the sphincter to close again. Hyperacidity tends to delay gastric evacuation, as do other intra-abdominal stimuli such as gall bladder or appendiceal reflex irritation.

Methods of Gastric Analysis

The older methods of gastric analysis, such as those of Töpfer and Rehfuss, have been shown to be of little value in most cases. One reason is because there are such wide variations in the quantity and acidity of the gastric secretion in normal people. With advancing age there is an increasing frequency of anacidity. Fully one-fourth of all healthy individuals over sixty years of age have practically no gastric secretion.

Studies of the gastric juice are helpful, however, in some conditions. If free hydrochloric acid is present in the gastric juice, pernicious anemia may be ruled out in a doubtful case. In suspected duodenal ulcer a high basal acidity is quite suggestive while on the other hand, a low acidity is high or not in all. formerly

believed.

For many years it has been customary to give some type of test meal with the idea of stimulating gastric secretion. Many objections to such a procedure may be raised. In addition to the dilution which is produced, the test meal also exercises a buffer action which tends to obscure the true state of secretory activity. The stimulus offered by the ordinary test meal is not sufficient to bring out the full capabilities of the secretory mechanism.

For these reasons the modern trend is to two types of procedure: (1) The measurement of basal gastric secretion obtained without artificial stimulus. (2) The examination of gastric juice obtained after stimulation with histamine.

Physical and chemical examination of the specimens secured by these methods gives all necessary information.

I. Test of Basal Gastric Secretion.—1. **Preparation of Patient.**—The patient is prepared as if for basal metabolism test. No food or drink is given for twelve hours and the patient is kept at rest.

2. **Procedure.**—Pass a gastro-duodenal tube of the Rehfuß type, cooled but not lubricated, for a sufficient distance to allow the tip to reach the most dependent part of the stomach. Maintain the position of the tube by strapping it to the angle of the mouth with adhesive. Have the patient expectorate all saliva into a basin or sputum cup; urge him not to swallow any. Emphasize this throughout the test.

With the tube in position aspirate all stomach contents using a 50-cc. Luer syringe. The fasting residuum usually amounts to 10 to 50 cc. of turbid, colorless or slightly bile-stained fluid with a variable admixture of mucus. If pyloric obstruction is present, the residuum will be much greater. Keep up continuous suction with the syringe, securing successive portions each ten minutes for an hour. After the first few ten-minute periods the secretion of gastric juice usually reaches a steady level. Keep each specimen in a separate tube, with proper identification.

The amount so obtained and its acidity are taken as the basal secretory value for the individual. Remarkably constant results are obtained on the same subject on successive days.

3. **Normal Values.**—(a) *Volume.*—In healthy individuals the amount secreted in a ten-minute period varies from 5 to 25 cc.

(b) *Acidity.*—The total acidity varies from 0 to 110, expressed as cc. of 0.1 N alkali required to neutralize 100 cc. of gastric contents. The free HCl usually requires 10 to 30 cc. less alkali (0.1 N) to neutralize it, per 100 cc. of gastric juice.

If any large amount of bile is regurgitated it will vitiate the results, requiring repetition of the test. As a rule this measurement of basal secretion suffices, but if there is no free acid present, the following procedure must be done.

II. Histamine Test of Gastric Function.—Of the various pharmacodynamic drugs used to promote gastric secretion, histamine is by far the best. It stimulates the greatest flow of juice of the highest acidity the stomach is capable of producing and has but few side effects. It produces a wheal at the site of injection and may cause hot flushes of the face and neck but there are no noteworthy constitutional symptoms.

1. **Preparation of Patient.**—As for the basal test.

2. **Procedure.**—Pass the tube and aspirate all fasting contents. Continue aspiration collecting the fasting secretions for two or three ten-minute periods, until they have reached a minimum.

Inject the proper dosage of histamine hydrochloride subcutaneously. The usual dosage is 0.01 mg. per kilo of body weight. A solution containing 1 mg. per cc. may be made. Stored in the refrigerator it will keep for several weeks.

Continue the aspiration for four to six ten-minute periods. Place each portion in a properly labeled tube.

Some practice is necessary before the aspirating procedure can be carried out satisfactorily. There is a tendency at first to traumatize the mucosa since it is difficult to make continuous aspiration during the alter-

nating phases of tone and relaxation to which the fasting stomach is subject. After a little experience a tactile sense is developed which makes the procedure easy.

3. **Normal Values.**—(a) *Volume.*—The amount obtained varies from 5 to 50 cc. in normal individuals. Only rarely is the latter figure exceeded. In the majority the maximum ten-minute amount varies from 15 to 40 cc.

(b) *Acidity.*—The highest total acidity varies from 0 to 160, expressed as the cc. of 0.1 N alkali required to neutralize 100 cc. of gastric fluid. In the majority of normal people it lies between 80 and 130.

4. **Sources of Error.**—It is possible that in spite of continuous aspiration all of the gastric contents may not be recovered in any ten-minute period. Also, some gastric secretion may escape through the pylorus. However, it is quite unlikely that any considerable amount would be lost in this manner.

If regurgitation from the duodenum occurs as evidenced by marked bile staining, the test is worthless and must be repeated.

The fluid aspirated is usually clear or slightly opalescent with more or less mucus which can be removed by centrifugalization. This fluid is suitable for all physical and chemical tests.

Physical Examination

1. **Macroscopic.**—1. *Quantity.*—The average adult's stomach has a capacity of approximately 1500 cc. The fasting residuum varies up to 100 cc. but rarely exceeds 50 cc. An increase may be due to retention caused by pyloric obstruction or spasm, or to regurgitation of duodenal contents. With obstruction the amount retained may be large, varying up to several liters, and containing particles of food eaten more than twelve hours previously. Duodenal regurgitation is recognized by bile staining of the contents.

2. *Emptying Time.*—The emptying time varies within wide limits but the normal stomach usually empties within seven hours after a light meal.

3. *Appearance.*—The normal fasting residuum may be thin, clear and colorless in those who have a continuous free flow of gastric secretion. However, as a rule it is turbid, colorless, gray or bile-tinged, with a greater or lesser admixture of masses of saliva and mucus. Some specimens are thin but mucoid or syrupy. Blood may appear as such but ordinarily in small amounts it is changed to a light or dark brown by the acid of the gastric juice. Larger amounts which have remained in the stomach for any period of time may have a "coffee grounds" appearance. Blood most often is the result of trauma from the tube but may come from cancer, ulcer or ruptured varicosities. In occasional cases pus or tissue fragments may be noted.

4. *Odor.*—Clear, fresh gastric juice has but slight odor. If much mucus or saliva is present there may be a slightly sour, musty odor. Only very rarely, with the sloughing of tissue, is the odor foul.

Chemical Examination

1. **Acidity Determinations.**—In both the basal and histamine tests it is customary to determine the total acidity and the free hydrochloric on the fasting residuum and on each ten-minute specimen.

1. **Reagents.**—(a) *Sodium Hydroxide*, 0.1 N solution.

(b) *Phenolphthalein Indicator*.—Dissolve 0.1 gm. of phenolphthalein in 100 cc. of 50 per cent ethyl alcohol.

(c) *Topfer's Indicator*.—Dissolve 0.5 gm. of p-dimethylaminoazobenzene in 100 cc. of 95 per cent ethyl alcohol.

2. **Procedure.**—(a) *Free HCl*.—Filter the specimen if necessary. Measure 5 cc. of specimen into a small porcelain evaporating dish or into a small beaker. Add a drop or two of Töpfer's reagent. If free HCl is present a bright red color is produced. Titrate with 0.1 N NaOH until the bright cherry red changes to a salmon pink.

(b) *Combined Acidity*.—Add to the specimen above, one drop of phenolphthalein indicator and continue the titration until a faint pink which persists for two minutes is produced.

3. **Calculations.**—(a) *Free Acidity*.—The number of cc. of 0.1 N NaOH used times 20 gives the free acidity value.

(b) *Total Acidity*.—The total amount of 0.1 N alkali used for both the free and the combined acidity multiplied by 20 gives the value for total acidity.

II. **Blood.**—Occasionally it may be necessary to examine a specimen for blood by chemical means. The benzidine method is one of the most sensitive provided the reagents are of satisfactory quality.

1. **Reagents.**—(a) *Benzidine*.—A saturated solution in glacial acetic acid. Stored in a dark brown bottle this solution keeps fairly well.

(b) *Hydrogen Peroxide*.—The usual 3 per cent solution. Since peroxide deteriorates rapidly it should be tested before use. Add a few drops of a solution of potassium dichromate in sulfuric acid to 2 cc. of the peroxide. A deep blue color indicates that the peroxide is still active.

2. **Procedure.**—(a) *Direct Test*.—To 3 cc. of benzidine solution add 2 cc. of gastric contents and mix. Add 1 cc. of hydrogen peroxide.

Discard the contents of the solution. Discard the ether extract. Make the residue slightly acid with acetic acid and again extract with ether. Evaporate the ether to dryness on a water bath. Dissolve the residue in 1 cc. of water, then add a few drops of the benzidine solution and a drop or two of hydrogen peroxide.

3. **Result.**—If blood is present a green to deep blue color develops, the depth of color depending on the amount. Too much benzidine or too much peroxide interferes with the delicacy of the test.

III. **Other Tests.**—Tests for lactic acid, gastric enzymes, protein, chlorides, etc., give no information of clinical value. They are no longer advised in routine work.

DUODENAL FLUIDS

Introduction.—The duodenal contents are a mixture of duodenal secretion, the pancreatic juice, bile and the chyme discharged from the stomach. The usual procedure includes macro- and microscopic examination, and tests for pancreatic enzymes. The fluid is removed from the duodenum by siphonage through a flexible tube. After removal of the residual contents the flow of bile is stimulated by injecting magnesium sulfate or other sub-

stance which relaxes the sphincter of Oddi, or by use of a physiologic cholagogic agent such as olive oil, peptone or oleic acid.

I. Collection of Specimens.—1. Introduction of the Tube.—The patient is prepared as for a test of basal gastric secretion.

The duodenal tube marked at 40, 56 and 70 cm. (indicating the distance from the lips to the cardia, the pylorus and the duodenum respectively) is introduced into the oropharynx. The patient is instructed to swallow, aided by mouthfuls of water if necessary. The tube may be gently urged past the glottis; once by this point it passes readily into the stomach. When mark II has reached the lips, aspirate the gastric residuum which may be examined as described under gastric analysis. The stomach is then washed out with water at body temperature until the return is clear. The proximal end of the tube is then clamped off; the further advancement of the tube is accomplished by the peristaltic action of the stomach; if the tube is forced it tends to coil in the stomach. After the tube has advanced to mark III, withdraw trial specimens of the contents. If the tube lies in the duodenum 5 or 10 cc. of a viscid alkaline liquid is obtained; if still in the stomach an acid liquid resembling the specimen of gastric residuum is aspirated. If the tube is still in the stomach, withdraw it gently back to mark II, and again allow it to find its way into the duodenum. As evidence of the position of the tube the following points may be noted: (1) If the end of the tube is in the stomach aspiration will find air or fluid and the rubber tube will not collapse. If it is in the duodenum, rapid aspiration will collapse the tube; on waiting a few minutes and aspirating slowly, fluid enters the syringe. (2) If air is injected through the tube into the stomach the patient feels its entrance plainly and can localize it. When the tube lies in the duodenum, the patient does not usually feel it. (3) If a stethoscope is held over the epigastrium and air is injected in quick spurts, bubbling sounds will be heard over a large area, with maximum intensity to the left of the midline, if the tube is in the stomach. If it lies in the duodenum the sounds are crepitant, less resonant and maximum in the right hypochondrium. (4) If, after drinking a half glass of water, the water can be immediately aspirated, the tip of the tube is in the stomach. (5) Fluoroscopic localization of the metal tip of the tube is the most accurate method.

2. Stimulation of Secretion.—When the tip of the tube is definitely in the duodenum inject a little air to distend the gut lumen, then gently aspirate the duodenal contents. To secure relaxation of the duodenum and to promote a flow of both pancreatic juice and bile one of the following agents is instilled through the tube:

(a) A saturated solution of magnesium sulfate is diluted with two volumes of water. This solution is warmed to body temperature and permitted to run down the tube by gravity, the barrel of the syringe being used as a graduate. Run in three portions of 25 cc. each at ten-minute intervals.

(b) *Peptone*.—Use 50 to 100 cc. of a 5 per cent solution which has been boiled and filtered.

(c) *Olive Oil*.—Use 15 to 30 cc. The addition of 15 cc. of hot water facilitates its introduction. The oil rises to the top of the specimens and does not dilute the bile. For this reason it is most satisfactory when quantitative chemical examination is desired. It may cause difficulty in

microscopic examination particularly to the inexperienced. "B" bile may be obtained by the use of olive oil when other procedures have failed.

(d) *Oleic Acid*.—Mix 5 to 10 cc. of chemically pure oleic acid with 15 to 30 cc. of warm water.

After instillation of the stimulating agent, pinch the tube to maintain siphonage and attach to a drainage bottle. Suction by a syringe or 1-ounce bulb is applied if the fluid does not run out by gravity. The tube should have a "glass window" inserted so that the flow of bile may be observed. As the bile alters in appearance the collection bottle should be changed. When no further bile is obtained or the required specimens have been secured, a little air is injected to balloon out the walls of the duodenum and to free the tip of the tube. The tube is then gently withdrawn, being temporarily arrested at the pylorus and glottis.

II. **Types of Bile from the Duodenum.**—1. The fluid first obtained when the tube reaches the duodenum is duodenal juice. It is labeled "D." Generally 10 cc. or less are secured; it is light amber or golden yellow and alkaline in reaction.

2. After the introduction of the magnesium sulfate or other agent, the first bile that follows is light golden yellow and probably comes from the common, cystic and hepatic bile ducts. It is labeled "A" and varies in amount from 5 to 30 cc.

3. As drainage continues, the bile deepens in color to dark golden yellow, yellow brown or olive green and has a viscid, syrupy consistency. It is derived from the gall bladder and averages 30 to 60 cc. in amount. The terminal portion of this bile is used for bacteriological study. This sample, labeled "B," is also selected for examination for pancreatic ferments as the deepest colored bile contains these ferments in highest concentration. The sample should be kept cold as steapsin is unstable at room temperature.

4. On further drainage a lighter, thinner, lemon or straw colored bile is obtained. This originates from the liver and may drain for several hours. It may amount to from 30 to 200 cc. Label the sample "C."

Examination of the Specimens

I. Macroscopic Examination.—1. **Opacity.**—Normally the fluids are clear or only faintly cloudy. Marked opacity in all specimens suggests cholecystitis, the microscopic picture of which is characterized by the presence of leukocytes persisting after magnesium sulphate has been added to the bile ducts.

2. Color.—Failure to obtain "B" bile suggests that the gall bladder is not storing or concentrating bile. Dark bile may originate from a dilated or inflamed gall bladder. In such cases the normal sequence of light to dark bile is absent. In the presence of gall stones, *e. g.*, gall stones.

3. **Blood.**—If there is more than a trace an ulcer may exist. Benzidine tests for blood are not reliable as duodenal contents or fresh bile may give positive results.

II. Microscopic Examination.—The specimens should be examined within a few minutes after their withdrawal as the ferments rapidly destroy cellular elements. The samples are centrifugalized and the sediments examined. The same precautions that are observed in the examination of

CHAPTER V

LIVER FUNCTION TESTS

By CLON J. GENTZKOW and HOWARD A. VAN AUKEN

INTRODUCTION

appreciate and its excretions, its cylinders and desquamations, its pathologic albumin and other strange substances as has the urine. But things are as they are, and until the specialist, with his little tubes, converts our inside into an outside most doctors will have to be content with perceiving biliary disturbance at one or several removes.

Now, how small a biliary disturbance can the clinician perceive? Circumstances alter that case very much. The liver is the most silent of organs of only a small

Since the quotation above is unfortunately all too true, an indirect approach must be made, and so for the measurement of hepatic function certain tests have been devised. These tests evaluate the function

of the organ is known from clinical, laboratory, or other studies, these tests may offer considerable help in determining the effectiveness of medical treatment, in pre- and postoperative management, and in prognosis.

the various types of liver malfunction. The multiplicity of liver functions, not all of which may be injured equally under any given set of conditions, and the great reserve and reparative powers of the organ, are the reasons why this is true.

Thus of the liver the situation here is not strictly comparable with that in disease conditions. In the latter case, all of the cells may be injured slightly and dysfunction of one or more kinds result, yet the cells may appear normal on histological examination. For this reason and also because of the greater sensitivity of some of the newer tests, the oft heard statement that liver function tests are positive only in the late stages of disease is not necessarily true. However, it must be emphasized that the results are

of the degree of depressed function. Thus, Am. Jour. Med. Sci., 170, 625, 1925.

the galactose tolerance is frequently depressed in acute hepatitis but is usually normal in obstructive jaundice, at least in the early stages. Characteristic changes may be noted in the cholesterol partition and phosphatase values in these two conditions. Differentiation between the different types of chronic jaundice cannot be made by means of hepatic function tests alone.

TYPES OF TESTS

All liver function tests fall naturally into two general divisions:

Those dependent upon the excretion of bile, such as

- I. Van den Bergh reaction.
- II. Icterus index.
- III. Azorubin S test.
- IV. Bromsulphalein tests.
- V. Bilirubin excretion.
- VI. Serum phosphatase determination.
- VII. Cholesterol and cholesterol esters determinations.
- VIII. Examination of duodenal fluid.
- IX. Cholecystography.

Those not dependent upon the excretion of bile, but

- I. Dependent upon the deaminating function, such as the determination of aminoacids, urea, and ammonia in the blood, and Millon's test for tyrosinuria.
- II. Dependent upon the detoxicating function—hippuric acid tests.
- III. Dependent upon glycogenic function—galactose, levulose and glucose tolerance tests.
- IV. Dependent upon prothrombin formation—prothrombin time.
- V. Dependent upon production of serum proteins—albumin-globulin ratio; blood fibrinogen determinations.

Not all of these tests are practical; some of them yield only limited information or become positive only in the late stages of hepatic disease; still others may be influenced too greatly by other conditions which may be present concurrently with liver disorders. For these reasons the tests in common use are comparatively limited in number.

In evaluating hepatic function it is necessary to employ a number of tests in order to reach conclusions as to the state of the organ and to avoid missing some cases which may show decrease in some activities but not in others. The concurrent use of the quantitative van den Bergh reaction, the bromsulphalein, hippuric acid and cephalin-cholesterol flocculation tests has been found to be most practical and to give the most satisfactory information except in the differential diagnosis of acute jaundice. In this latter instance, the concurrent repetitious use of the galactose tolerance test, and the determination of the cholesterol partition, the bilirubin content, and the phosphatase level of the blood are most helpful. Repetition of these tests three times in the first week of observation will usually be necessary.

TESTS OF LIVER FUNCTION CONCERNED WITH BILE EXCRETION

An understanding of these tests requires a knowledge of the physiology

endothelial system of the spleen, liver and bone-marrow, the hemoglobin being split into bilirubin and a colorless iron residue. Both of these substances are excreted into the blood stream, but the fate of the iron containing residue is unknown. The bilirubin in the blood normally lies between 0.2 and 0.25 mg. per 100 cc. The polygonal cells of the liver remove a part of the bilirubin from the blood and excrete it through the bile ducts into the duodenum. From the intestines the excreted bilirubin may be reabsorbed, but most of it is reduced by the action of the intestinal bacteria to a colorless compound, urobilinogen, which in turn is oxidized to urobilin (stercobilin). This passes out in the feces; some of it is reabsorbed into the blood. In

It was formerly believed that in its passage through the liver cells a physical change occurred in bilirubin and that this change was reflected in the type of van den Bergh reaction resulting. As formed by the reticulo-endothelial cells, bilirubin was supposed to be a colloid, which in turn was converted into a crystalloid in its passage through the polygonal cells of the liver.

The prompt direct van den Bergh reaction was believed to be due to the crystalloid form and the delayed direct reaction to bilirubin undergoing transition from the colloid to the crystalloid state, the length of the delay indicating the degree of change which had occurred.

Later work, however, seemed to indicate that the variation in reaction was related only to the actual amount of bilirubin present in the blood. Another theory devised to account for the difference in reaction postulated the presence of catalytic or inhibiting substances which caused varying percentages of the total bilirubin present to react with the diazo reagent.

More recently the development by Evelyn and Malloy¹ of an accurate quantitative method for the determination of bilirubin in the blood, using the photoelectric colorimeter, has resulted in studies disproving the older

that all bilirubin in action is attached to plasma albumin as a dissociable complex, while that which does not give the direct reaction is attached to a definite fraction of the albumin, probably by a valence bond.

van den Bergh reaction; (2) an estimation of the degree of the jaundice by comparing the intensity of color of normal and icteric sera. This is the icterus index.

I. Ring Test (Lepehne, 1921; Elton, 1930).—1. *Reagents*.—(a) *Ehrlich's Diazo Reagent*.—As described under the van den Bergh reaction, page 69.

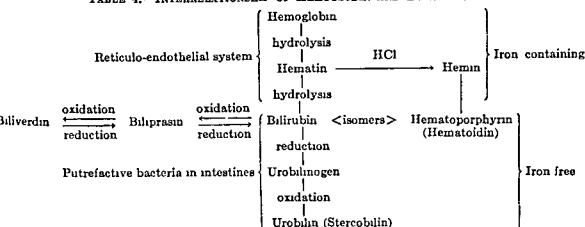
2. *Method*.—Place 1 cc. of serum in a Wassermann or Kahn tube. Slant the tube and overlay with 0.5 cc. of the diazo reagent. Examine the contact zone of the two fluids for the development of a red ring. If no color appears within sixty seconds, gently shake the tube, lowering the

¹ Jour. Biol. Chem., 132, 119, 1940.

² Jour. Biol. Chem., 119, 481, 1937.

contact zone, but allowing a small amount of serum to remain undisturbed as a control. After mixing, observe for at least ten minutes for development of a red, amber or port wine color. The tube is best observed by natural daylight, preferably against a glazed window.

TABLE 4.—INTERRELATIONSHIP OF HEMOGLOBIN AND DERIVATIVES



II. Van den Bergh Indirect or Quantitative Reaction (Tannhauser and Andersen, 1921).—Practically all clinicians and clinical pathologists now regard the direct or qualitative van den Bergh reaction as valueless. Attempts at correlating the different resultant reactions with the various types of jaundice and disease conditions producing icterus have failed.

At the present time most workers prefer the actual determination of the bilirubin in serum or plasma. An accurate method, that of Evelyn and Malloy, is available but requires the use of a photoelectric colorimeter or spectrophotometer. This method is given on page 214.

No accurate method using the visual colorimeter has been found to date. The indirect van den Bergh remains as the best method of estimation by visual colorimetric means, but it should be remembered that it is only approximate, an estimation not a determination. In the original van den Bergh method, the alcohol was added before the diazo reagent, resulting in some loss of pigment due to the precipitated albumin carrying it down. Tannhauser and Andersen avoided this by adding the diazo reagent first, thus permitting the coupling of diazo and bilirubin. The azobilirubin formed is more soluble in, and more completely extracted by the alcohol.

1. **Reagents.**—(a) *Solution A.*—Dissolve 1 gm. of sulfanilic acid in 15 cc. of concentrated hydrochloric acid. Dilute to 1000 cc. with distilled water. This solution should be prepared freshly every two weeks.

(b) *Solution B.*—Dissolve 0.5 gm. of sodium nitrite in 100 cc. of distilled water. It should be freshly prepared every two weeks.

(c) *Solution A-B or Diazo Reagent* (Ehrlich's Reagent).—This solution is made immediately prior to performing the test, by mixing 10 cc. of solution A with 0.3 cc. of solution B.

(d) *Ethyl Alcohol*, 95 per cent.

(e) *Ammonium Sulfate*, Saturated Solution.

(f) *Standards.*

(1) *Cobalt Sulfate* (McNee and Keefer, 1925).—Dissolve 2.161 gm. of anhydrous cobaltous sulfate which has been dried to constant weight at 105° C., in distilled water and dilute to 100 cc. If crystalline cobaltous

sulfate ($\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$) is used, dissolve 3.92 gm. in 100 cc. of water. This standard is equivalent to one van den Bergh unit. It is permanent if kept protected from light. The addition of 0.5 cc. of concentrated sulfuric acid per 100 cc. adds to the permanency of color.

(2) Cobalt Sulfate Standard (White, 1932).—White changed the standard of McNee and Keefer so that it would be equivalent to 0.1 mg. of bilirubin per 100 cc. of serum. This is nearer the true value of the van den Bergh unit, the previous figure being 20 per cent too high.

Dissolve 13 gm. of anhydrous cobaltous sulfate in 50 cc. of water. Add gradually 40 cc. of concentrated HCl. Shake, keeping the solution cool under running water. Dilute to 100 cc. Allow to stand twenty-four hours prior to use. It will keep for months in a well-stoppered bottle in the dark.

2. Procedure.—Pipet 1 cc. of fresh unhemolyzed serum into a 15-cc. graduated centrifuge tube. Add 0.5 cc. of diazo reagent. After two minutes add 2.5 cc. of 95 per cent ethyl alcohol and 1 cc. of saturated ammonium sulfate. Mix thoroughly by inverting several times. Centrifugalize at moderate speed for five minutes. A sharp division into three layers is obtained. The bottom layer is the ammonium sulfate solution, the middle layer is the alcohol-soluble proteins, the top layer being the unextracted bilirubin. The top layer which is clear or slightly cloudy and colored pink to deep violet. Measure the volume of this colored layer by the graduations on the tube; it is usually 2.5 to 3 cc. Pipet this layer into a colorimeter cup and compare with the standard. If the colored layer is cloudy, heat in warm water. If the color of this alcoholic extract is too deep to match the standard, accurately dilute it with 60 per cent alcohol in proportions of 1 to 2 to 1 to 10 as required. Occasionally a yellow or orange color in the alcoholic layer makes it difficult to compare against the standard. A few drops of oxalic acid usually changes the color back to pink but may give turbidity. Turbidity may be removed by adding a few drops of 10 per cent sodium carbonate solution and 1 additional cc. of alcohol. If the latter is added, it must be included in the calculation of the alcoholic layer in the calculation. If the color of the standard may be compared with the color of the standard may be compared with the standard is diluted accurately with water.

3. Calculation.—This method is an estimation of bilirubin. There is no agreement as to the dilution factor. Tannhauser and Andersen considered the dilution to be 1 to 6, whereas actually it is 1 to 3 by their method as all the bilirubin extracted is in the alcohol layer which normally is about 3 cc., although it varies with each test. For greater accuracy use the volume in cc. of the alcoholic layer as the dilution factor.

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{\text{volume in cc. of alcoholic layer}}{\text{volume in cc. of standard}} \times \frac{\text{dilution if any with 60 per cent alcohol}}{\text{dilution of standard}} = \text{number of van den Bergh units}$$

Number of units $\times 0.5$ (value in mg. of one unit using the McNee and Keefer Standard) = mg. of bilirubin per 100 cc. serum. If the White standard is used, the units $\times 0.4$ = mg. bilirubin per 100 cc. of serum.

If the standard is diluted in order to obtain a more accurate match with the faint colors of the low normals, the results obtained in the above equations must be divided by the dilution of the standard.

4. **Results.**—(a) *Normal.*—The alcohol layer has a faint pink color. Values of 0.2 to 0.25 mg. of bilirubin per 100 cc. of serum are found. Bilirubin is increased during prolonged fasts; it decreases following ingestion of food, being lowest two to six hours after a meal.

(b) *Abnormal.*—Infants show a physiological hyperbilirubinemia due to the high rate of red blood cell destruction in the first few weeks of life. In the majority of individuals values over 0.5 mg. per 100 cc. are considered abnormally high. Up to 1 mg. per 100 cc. is considered the zone of "latent jaundice." Highest values are present in obstructive jaundice.

III. Icterus Index.—This test is based on the comparison of the intensity of the yellow color of the serum, against an arbitrary standard potassium dichromate solution.

1. **Reagents.**—(a) *Standard Potassium Dichromate Solution.*—In a liter volumetric flask place 0.1 gm. of potassium dichromate. Dissolve in about 500 cc. of distilled water. Add 2 drops of concentrated sulfuric acid and fill to the mark with distilled water. This 1 to 10,000 solution represents an arbitrary index of 1. It is paler than normal serum. It keeps six months in the dark.

(b) *Sodium Chloride Solution.*—Dissolve 0.9 gm. of sodium chloride in 50 cc. of distilled water in a 100 cc. volumetric flask. Dilute to the mark with distilled water.

2. **Procedure (Meulengracht-Bernheim Method).**—Withdraw 5 to 10 cc. of blood from an arm vein, using a dry needle, syringe and centrifuge tube to prevent hemolysis.

Depending on the intensity of its color, the serum is accurately diluted with the sodium chloride solution, so as to approximate the color of the standard, thus permitting better matching.

Place the standard potassium dichromate solution in the left hand cup of the colorimeter, which is set at 15 mm. Place the serum in the right cup, and make the comparison.

3. **Calculation.**—

$$\frac{\text{Standard reading (15)}}{\text{Unknown reading}} \times \begin{matrix} \text{dilution of serum} \\ \text{if any} \end{matrix} = \text{icterus index.}$$

The figure obtained indicates that the color of the serum is that many times the intensity of the color of the arbitrary standard.

4. **Method Using Block Comparator.**—Where a colorimeter is not available, a rough but fairly accurate estimation can be made by comparing the color of the serum against a standard set of tubes, containing dilutions of a stock potassium dichromate solution (1 to 100) corresponding to varying icterus indices. As one of the chief values of the determination is the detection of latent jaundice, two series of standard tubes should be available, one set including the indices between 1 and 20, the other between 20 and 100. Due to the intensity of the yellow color in the higher standards, color comparison is difficult with the error increasing as the saturation point is approached.

The standards are prepared as shown in Table 5. They are placed in clear serological test tubes, 100 × 10 mm. and sealed. A similar tube containing the serum is compared against the standard set. If the color of the serum is too intense, dilute with 0.9 per cent sodium chloride solution,

in a definite ratio, 1 to 2 to 1 to 5. This will bring the color of the serum in the more readable range (1 to 20) of the standard tubes. The index of the standard tube matched by the color of the serum, multiplied by the dilution of the serum, equals the icterus index.

TABLE 5.—PREPARATION OF STANDARD TUBES

Tube	Cc. of dichromate stock solution, 1 to 100	Cc. of distilled water containing 2 drops of concentrated H_2SO_4 to 100 cc.		Icterus index
1	5 0	5 0		50
2	4 5	5 5		45
3	4 0	6 0		40
4	3 5	6 5		35
5	3 0	7 0		30
6	2 5	7 5		25
7	2 0	8 0		20
8	1 6	8 4		16
9	1 4	8 6		14
10	1 2	8 8		12
11	1 0	9 0		10
12	0 9	9 1		9
13	0 8	9 2		8
14	0 7	9 3		7
15	0 6	9 4		6
16	0 5	9 5		5
17	0 4	9 6		4
18	0 3	9 7		3
19	0 2	9 8		2
20	0 1	9 9		1

5. Interpretation of Results.—(a) *Normal Zone*.—It lies between 4 and 7, corresponding to less than 1 van den Bergh unit.

(b) *Subnormal Zone*.—It lies between 2 and 4. It is usually associated with a secondary anemia of the hypochromic microcytic type, found in hemorrhage and malignancy, when not accompanied by passive congestion or obstruction of bile excretory passages. In contrast, the primary anemias of pernicious type, during their active phase, have an index of 7 to 20.

(c) *Latent Jaundice Zone*.—It lies between 7 and 18, corresponding to 1 to 4 van den Bergh units. It indicates a hyperbilirubinemia, without clinical evidence of jaundice in the tissues or the urine. An increased icterus index occurs when there is obstruction to the bile ducts, or hepatic insufficiency, or an excessive bilirubin production that accompanies increased red blood cell destruction. This last occurs in the various diseases associated with a hemolytic jaundice. In the milder toxic and infectious types of hepatitis, such as accompany lobar pneumonia, septicemia, chronic cardiac failure, passive congestion of the liver, mild arsenical poisoning, cholecystitis and cholelithiasis, the icterus index usually lies in this zone. An increased icterus index is a warning to stop arsenical treatment in syphilis.

(d) *Frank Jaundice Zone*.—It lies over 18, corresponding to 4 or more van den Bergh units, and may reach 150. Jaundice is obvious in the body tissues and fluids, including the urine. Such values are found most often in the later stages of chronic obstruction to the bile passages, in severe cholangitis, in severe generalized hepatitis such as acute yellow atrophy, and in severe poisoning caused by arsenicals, chloroform, carbon tetrachloride, and phosphorus. The icterus index drops rapidly in the first twenty-four hours after a successful operation for the removal of the cause of the obstruction.

IV. Bromsulphalein Tests.—The value of dye elimination tests in estimating renal function prompted the search for similar tests for the estimation of liver efficiency. Such a dye was discovered in phenoltetrabromphthalein sodium sulfonate. The test as originally proposed involved the intravenous injection of 2 mg. of the dye per kilogram of body weight and estimation of the quantity of dye retained at the end of thirty minutes. Recently the use of 5 mg. of dye per kilogram has been suggested as detecting liver damage earlier than the older test. Normal standards have been corrected so that the tests are much more sensitive. Serial determinations of dye retention have been suggested though these do not appear to offer any great advantage and are technically more difficult.

1. Dye Preparation and Dosage.—The dye is put up in a 5 per cent solution; each cc. contains 50 mg. The usual dose is body weight. The weight of the patient in the number of cc. of the 5 per cent solution 2 mg. per kilogram dosage is to be administered divide the weight of the patient by 55. No preparation of the patient is required, but to avoid postprandial clouding of the serum, which would interfere with accurate colorimetric comparison, overnight fasting is preferable.

2. Procedure.—(a) *Administration of Dye.*—Take a 5 cc. blood sample as a control. The icterus index and van den Bergh determinations may also be made on this specimen. Have ready a graduated glass syringe containing the proper amount of the dye; inject the dye solution through the needle already in place, taking one minute for the injection. Inject a few cc. of physiological saline to flush out the remaining dye. Avoid extravascular leakage; if such occurs, it is better to stop further injection at that site. Note the exact time at which the injection is completed.

(b) Exactly five minutes after completing the injection withdraw the second 5 cc. blood specimen from a vein in the opposite arm.

The third blood specimen is taken forty-five minutes after the injection if the 5 mg. per kg. dosage was used; at twenty minutes if the 2 mg. dose was given. These times are shorter than originally proposed but it has been shown that the normal liver eliminates all of the dye in this time. Some workers omit the five-minute specimen, taking blood thirty and lowed to eted into 10 mm.). clear it.

To the other sera add 2 drops of 10 per cent sodium hydroxide. If dye is present a red-violet color develops in the alkalinized sera, the intensity indicating the concentration of the dye present.

3. Standard.—A stock standard representing 100 per cent concentration is made by dissolving 10 mg. of dye for the 5 mg. dose or 4 mg. for the 2 mg. dose in 100 cc. of distilled water, alkalinized by the addition of 0.25 cc. of 10 per cent sodium hydroxide. From this stock standard a series of lesser standards are made, varying from 5 to 90 per cent. As the majority of the values ordinarily encountered fall below 50 per cent, the standards between 5 and 50 are made up at 5 per cent intervals; the standards over 50 are spaced at 10 per cent intervals. In making the dilutions, use water to which 0.25 cc. of 10 per cent sodium hydroxide per 100 cc. has been added.

driven off. After cooling, the preparation is ready for use. The emulsion should be prepared just before use in order to obtain comparable results.

2. Procedure.—Place 0.2 cc. of the serum in a centrifuge tube, add 4 cc. of normal saline and 1 cc. of the cephalin-cholesterol emulsion. Shake thoroughly, stopper with cotton and set aside at room temperature. The undisturbed tube is observed at twenty-four and forty-eight hours for flocculation.

Precautions—The serum used should be fresh or preserved in the refrigerator. Plasma is not very satisfactory as the anticoagulants used seem to interfere. Clean glassware must be used as heavy metals or strong acids may cause precipitation.

3 *Results and Interpretations.*—A negative test is one in which no flocculation occurs. A ++++ reaction is one in which there is complete flocculation. Varying degrees of precipitation are recorded as +, ++, or +++ A negative reaction should not be recorded with less than twenty-four hours observation.

The test is almost never positive in normals; occasionally a single plus reaction is obtained. It is also negative in cases of obstructive jaundice as a rule. In those cases in which precipitation does occur the reaction is usually slight, generally in cases of long standing in which some damage to the liver parenchyma has probably occurred. The test may fail in arsphenamine hepatitis.

The emulsion is flocculated in those cases showing active disturbances of the liver parenchyma, the degree of flocculation roughly paralleling the severity of the process. The test has considerable prognostic value; a decreasing flocculation indicating recovery, a persistently complete precipitation being of grave import. Single or circumscribed lesions in the liver usually give negative results as does hemolytic jaundice. On the other hand, the jaundice accompanying acute or chronic infections such as pneumonia or septicemia are characterized by positive tests.

V. *Miscellaneous.*—1. *Protein Determinations.*—Inasmuch as at least a large portion, if not all, of the serum albumin and globulin are produced in the liver, measurement of these substances may be indicative of liver damage particularly if other causes for change can be ruled out. Reduction in both fractions may be expected with frequent inversion of the albumin-globulin ratio. The degree of variation from normal roughly parallels the degree of hepatic impairment. For methods of determining the proteins of the blood, see Chapter XVI.

2. *Erythrocyte Size.*—The size of the erythrocytes in hepatic disease may be altered since the liver elaborates a substance which is concerned with normal red blood cell production. In damage to the parenchyma of the liver, there may be an average macrocytosis of 8.8 microns or more.

CHAPTER VI

THE FECES

By CLEON J. GENTZKOW and HOWARD A. VAN AUKEN

NORMALLY the feces consist of an innumerable variety of substances—water, undigested and unabsorbed food residues, intestinal secretions which have not been destroyed nor re-absorbed, bile, salts of calcium, iron and other metals, bacteria and their decomposition products, epithelial cells from the intestinal wall, pigments and mucus.

Abnormally, one may find increased amounts of any of the normal constituents, gall stones, pancreatic calculi, enteroliths, blood, pus, serum, parasites and their ova, and pathogenic bacteria.

Much information of importance can be gained by careful inspection of the fresh whole stool plus a few simple tests. All too frequently the only examination made consists of a search for parasites or their ova. On the other hand, routine complete examination of the feces is neither feasible nor desirable. When indicated, many special procedures are available.

The stool should be collected in a clean container. Examination should be made as promptly as possible and in any case, should not be delayed more than a few hours because of the changes due to decomposition. The odor may be partially controlled by adding a little 5 per cent phenol or formaldehyde. These substances cannot be used when a search for parasites or bacteria is to be made. In sending feces to the laboratory the jar should not be filled more than one-tenth to one-fourth; this will allow for the expansion of gases. Sterile containers must be used in collecting specimens for bacteriological examination.

MACROSCOPIC EXAMINATION

I. **Consistency and Form.**—The stool is normally soft but formed. In constipation one usually finds dry, hard masses (scybala). After cathartics and in diarrheas and dysenteries, the consistency may be mushy, liquid or watery, depending on the severity of the purgation. Flattened or ribbon-like stools may be found in spastic colitis, obstruction or stricture of the rectum or anus.

II. **Quantity.**—The average amount varies greatly but usually is about 100 to 170 gm. daily. The dry weight averages 24 to 45 gm. On a vegetable diet the quantity is greatly increased.

III. **Odor.**—The normal offensive odor is due chiefly to indol and skatol. The diet taken greatly affects the odor, which is most marked on a meat diet, less so on a vegetable and slight on a milk diet. A sour odor due to fatty acids is normal in infants. Putrid odors may be noted in childhood diarrheas. A very foul stench in adults suggests malignant or syphilitic ulceration of the rectum or gangrenous dysentery.

IV. **Color.**—Diet and drugs have a marked influence upon the color of the feces and must be considered in evaluating the pathological significance of abnormal colors. The normal brown color is due chiefly to the bile pigment hydrobilirubin (urobilin), a reduction product of bilirubin.

TABLE 6—THE COLOR OF FECES

Color	Diet and drugs	Pathological
Yellow	Milk, cornmeal, rhubarb; senna, santonin	Unchanged bilirubin
Green	Spinach and other green vegetables, calomel	Biliverdin, or rarely due to green-producing bacteria
Gray (clay color)	.	Absence or deficiency of bile, presence of excess of fats
Dark red-brown—chocolate color	Coffee, chocolate, cocoa, blackberries, cherries	
Red	Beets, tomatoes, apple skins, etc	Blood from rectum, anus or colon
Black	Charcoal, bismuth, iron	Altered blood from upper gastrointestinal tract, "tarry stool"
Colorless or watery with flakes of epithelium		Absence of bile pigments due to rapid passage of great quantities of fluids as in Asiatic cholera

V. **Mucus.**—Mucus in visible quantities is always abnormal and indicates intestinal irritation. When accompanied by pus it indicates inflammation or ulceration of the bowel. A variable amount of blood is usually associated with mucus. Intestinal mucus is most abundant in the dysenteries (amebic and bacillary), and in such conditions as mucous colitis, chronic ulcerative colitis and cancer of the large bowel.

VI. **Helminths.**—The larger helminths are detected by simple inspection or by washing the stool through a coarse sieve with tap water. Artifacts such as banana fibers, shreds of undigested vegetables, fruit skins, ribbons of mucus, etc., must not be mistaken for animal parasites. The larvæ of flies are occasionally found in the feces during the summer months as accidental contaminants.

VII. **Concretions.**—True gall stones are sometimes found if a search is made of the washed and strained stool. They usually show faceted surfaces and can be identified by crushing and testing them for bile pigments and cholesterol. Pseudo gall stones may be found after ingestion of large quantities of olive oil. They consist of fats and soaps.

MICROSCOPIC EXAMINATION

Viewed through the microscope the feces present an almost infinite variety of structures, representing end products of digestion. Care must be exercised not to mistake such objects as vegetable hairs and fibers, the husks of cereal grains, starch granules, the spores of ingested fungi, muscle and fibrous tissue, and other food remnants for parasites, ova or cysts.

For the special technic used in the identification of animal parasites, the reader is referred to the chapters on Protozoology and Helminthology. Methods for bacteriological examination are given in Part IV.

I. **Technic.**—1. **Fresh Whole Stool.**—Make a uniform emulsion of feces in water. Transfer a drop of the mixture to a glass slide and cover with a cover glass. The emulsion should be thin enough to render ordinary print legible when viewed through the preparation. Most of the routine examination can be made with such fresh preparations. Make a quick survey of the entire smear, using the low power of the microscope. Look for small helminths, motile protozoa, ova, cysts, muscle fibers, fat globules, crystals, pus and epithelial cells. The high dry power can then be used for the identification and study of selected objects. If the preparation is found

to be too thick and opaque it can be thinned by applying a piece of blotting or filter paper to the edge of the cover slip and absorbing excess fluid.

Some information as to the degree of digestion of fats, proteins and starches can be gained by microscopic examination of the feces after treatment with the following solutions:

(a) *Sudan III*.—Globules of neutral (undigested) fats and masses of fatty acids stain red.

(b) *Acetic Acid*, 30 per cent.—This brings out in sharp detail the structure of muscle and fibrous tissue. Muscle fibers show a progressive loss of cross striation and a rounding of the ends as digestion proceeds.

(c) *Lugol's Solution*.—Iodine will stain undigested starch granules blue; partially digested starch takes a reddish color with this solution.

2. **Examination for Exudate (Callender).**—(a) *Fresh Specimen*.—Examine a fresh loopful of material taken from bits of mucus, shreds or the blood-streaked portion of the feces. If none of these are visible macroscopically, collect exudate by stirring the liquid or emulsified stool vigorously with a wooden applicator. Exudate will cling to the applicator.

(b) *Stained Preparations*.—Mix thoroughly material obtained as above with equal parts of Loeffler's alkaline methylene blue, or with brilliant cresyl blue. Place a cover glass over the preparation and study under both low power and high dry lenses. Old leucocytes and macrophages stain quickly; young leucocytes stain slowly and show a peripheral deep-staining ring at the nuclear membrane. Red blood cells stain only after some minutes. Trophozoites of *E. histolytica* do not stain at first but after some minutes do pick up the dye. Cysts are more resistant. Bacteria stain promptly. Since *E. histolytica* loses its motility quickly, search should be made for trophozoites in the fresh, unstained preparations.

(c) *Permanent Preparations*.—To determine the species of amebae and whenever a permanent preparation is desired for other purposes, fix the specimen in sublimate-alcohol according to standard histologic technic. Stain in Harris' hematoxylin and eosin to study cytology and by one of the iron-hematoxylin methods to differentiate protozoa.

The technic outlined in (a) and (b) above is of great value in differentiating yeasts, which unstained, simulate leucocytes, and in making sure whether or not infection is present as determined by the presence and character of the exudate.

CHEMICAL EXAMINATION

I. **Determination of Reaction.**—The reaction of the feces is usually alkaline when freshly passed, but it may be slightly acid particularly on a carbohydrate diet. Intestinal indigestion may alter the reaction. After thoroughly mixing the sample of feces, the reaction may be determined with a good grade of litmus paper.

II. **Blood.**—The presence of blood in the stool may be evident on macroscopic examination; the stool may be bright red, dark red or black and tarry when blood is present in gross amounts. When present in traces only, chemical tests are necessary to detect the occult blood. In testing the feces for small amounts of blood, caution must be exercised in interpreting results unless the patient has been on a meat-free diet for two or three days.

A
feces

by ~~the~~ over it. The intensity of blue color and the speed with which it develops are a rough measure of the amount of blood present.

B. Guaiac Test.—1. Reagents.—*Gum guaiac, ether, glacial acetic acid and hydrogen peroxide.*

2. Procedure.—Boil a thin watery suspension of feces for a few minutes in a test tube. Extract the fat by shaking with an equal volume of ether. Allow the ether to separate and discard it. To the remainder, add one-third volume of glacial acetic acid and mix. Extract the acidified suspension again with one volume of ether. Allow to separate and to the ether extract add a few granules of gum guaiac. Mix well and add a few cc. of hydrogen peroxide.

3. Result.—A violet or blue color develops if blood is present.

C. Phenolphthalin Test (Gettler and Kaye¹).—The authors have reintroduced this test and have determined that its specificity is greater than that of the guaiac or benzidine tests and that its sensitivity is at least as great if not more so.

1. Reagents.—(a) *Phenolphthalin Reagent.*—Dissolve 20 gm. of sodium hydroxide and 1 gm. of phenolphthalein in 200 cc. of water in a 500-cc. Erlenmeyer flask. Add 20 gm. of granulated zinc, 20- to 30-mesh, connect with a reflux condenser and slowly boil until the red color of the alkaline phenolphthalein disappears leaving a colorless or faintly yellow solution. This may require two to three hours. Store in a brown glass, rubber-stoppered bottle in a cool, dark place. The reagent will keep for many months. If some of the granulated zinc is left in the solution it aids in keeping it in the reduced form.

(b) *Hydrogen Peroxide, 3 per cent.*

2. Procedure.—Boil 1 or 2 cc. of urine, gastric contents or thin fecal suspension for thirty seconds to destroy any oxidases present. Cool, then add 5 drops of phenolphthalin reagent followed by 3 drops of hydrogen peroxide.

3. Result.—A pink to red color indicates the presence of blood, the intensity of color being an indication of the amount. The color may fade after three minutes.

III. Bile.—The brown color of the feces is due to the presence of urobilin, a derivative of bilirubin, which in turn is derived from hemoglobin. Neither bilirubin nor hemoglobin normally appear in the feces. Estimation of urobilin is often helpful in distinguishing the anemias due to excessive hemolysis from other forms and in following the progress of pernicious anemia. The amount of urobilin in the feces is, however, subject to considerable variation due to constipation and other factors. It is absent in obstruction of the bile ducts. Its return indicates clearing of the obstruction.

Examination for bile is usually limited to tests for the pigments, the majority of the tests being based upon the oxidation of the pigments with the formation of a series of colored derivatives.

A. Schmidt's Test for Urobilin.—1. Reagents.—*Mercuric Chloride, saturated aqueous solution.*

¹ Gettler, A. O., and Kaye, Sidney, *Am. J. Clin. Path., Tech. Sect.* 7, 77, 1943.

2. **Procedure.**—In a white porcelain dish, rub up a small portion of feces in the mercuric chloride solution and allow this to stand for six to twenty-four hours.

3. **Results.**—The presence of urobilin is indicated by a deep red color which is imparted to the particles containing this pigment. If unaltered bilirubin is present in any portion of the stool that portion will be green in color due to the oxidation of bilirubin to biliverdin.

B. **Gmelin's Test for Bilirubin.**—This consists in bringing slightly yellow nitric acid into contact with a watery extract of feces. A play of colors, green and violet being most prominent, indicates the presence of bilirubin. Colorless nitric acid will become yellow upon standing in the sunlight. The test may be applied in various ways; by overlaying the acid with a suspension of the feces to bring out a colored ring; by bringing a drop of each together on a porcelain plate, or by filtering the fecal suspension through thick filter paper and touching the wet paper with a drop of the acid.

C. **Quantitative Determination (Watson).**—1. **Reagents.**—(a) *Ferrous Sulfate Solution*, 20 gm. of ferrous sulfate dissolved in 92 cc. of distilled water.

(b) *Sodium Hydroxide*, 10 per cent solution.

(c) *Modified Ehrlich's Reagent*. Dissolve 0.7 gm. of paradimethylaminobenzaldehyde in 150 cc. of concentrated HCl and add 100 cc. of distilled water.

(d) *Sodium Acetate*, saturated aqueous solution.

(e) *Glacial Acetic Acid*.

(f) *Petroleum Ether (Petroleum Benzine)*.

2. **Procedure.**—Thoroughly grind a 10-gm. portion of a well mixed 4-day collection of feces in a mortar with a few cc. of distilled water and dilute to 300 cc. or only to 100 cc. in obviously acholic feces. Add 100 cc. of the ferrous sulfate solution and then 100 cc. of the sodium hydroxide with constant stirring. Cork the flask and allow to stand one hour. To 2 or 3 cc. of the filtrate add an equal amount of the Ehrlich's reagent and then several cc. of the sodium acetate. Depending upon the intensity of the color developed, from 1 to 50 cc. of the filtrate are used in the quantitative procedure. Thus if the color is intense 1 cc. or even less will suffice; if pale red from 5 to 10 cc.; if faint 25 cc.; if absent 50 cc. It is desirable that the quantity of filtrate chosen should be only enough so that the final colored solution does not exceed 100 cc. and that its color is not more than half the intensity of the standard.

Place the quantity of filtrate in a separatory funnel, dilute to 20 cc. with water if less than this amount of filtrate was used, cover with 30 cc. of purified petroleum ether, strongly acidify with glacial acetic acid and shake vigorously. Separate and extract twice more with petroleum ether. Combine the ether, wash with water, and shake vigorously with 1 or 2 cc. of the Ehrlich reagent. At least twice as much of the sodium acetate solution is added, the maximum color now appearing. Separate the colored solution into a graduated cylinder and repeat the extraction until the urobilinogen is entirely removed. Make up the combined colored solutions to a volume convenient for calculation and mix. Pour out enough to fill the right hand test tube of a Helge-Dunning colorimeter (obtainable from Hysan, Westcott and Dunning). Should the intensity of the color

be greater than the 50 per cent standard, dilute further as comparisons are most accurate between 20 per cent and 50 per cent. It may be desirable to standardize the colorimeter with crystalline urobilinogen.

3 Calculation and Interpretation.—

$$\frac{\text{cc. of ferrous hydroxide mixture}}{\text{grams of feces used}} \times \frac{\text{cc. of final colored solution}}{\text{cc. of filtrate used}} \times \frac{1}{\text{No. of days collection of feces}}$$

$$\times \frac{\text{weight of 4-day quantity of feces}}{100} \times \text{percentage of color standard} = \text{mg. of urobilinogen per day.}$$

Example:

$$\frac{500}{10} \times \frac{50}{2} \times \frac{1}{4} \times \frac{300}{100} \times 0.20 = 187.5 \text{ mg. per day}$$

Normal individuals excrete 40 to 200 mg. with an average of 100 to 250 mg. of urobilinogen daily. Inanition, inactivity or low grade fever decrease the amount excreted. Hemolytic jaundice, pernicious anemia, Hodgkin's disease and leukemia increase the quantity. In some secondary anemias it may be reduced.

IV. Fats.—The stool contains variable quantities of neutral fats, fatty acids and soaps. Excess of fats is found in pancreatic disease, biliary obstruction and sprue. Neutral fats are present in very small amounts or not at all on ordinary diets.

A. Qualitative Differentiation.—Table 7 gives a simple method for differentiating these fatty elements of the stool:

TABLE 7.—TESTS FOR FATTY ELEMENTS IN FECES. (FROM KOLMER AND BOERNER.)

Test	Neutral fats	Fatty acids	Soaps
Microscopic appearance	Round or irregular globules; highly refractile or minute needles	Sheaves of large needles or short, delicate, curved needles which occur in such thick masses that the shape of the individual crystals can seldom be made out	Needles arranged in clusters or in fans or in short plump crystals or scales, in amorphous forms as gnarled bodies everted like the pinna of an ear; soap crystals are comparatively coarse, as a rule (thick, short needles or flakes), but may be indistinguishable from those of fatty acids
Heat	Melted	Melted	Not melted
Ether solubility	Dissolved	Dissolved	Not dissolved
Scharlach R*	Stained	Crystals not stained, globules stained	Not stained
Sudan III*	Stained	Crystals not stained; amorphous flakes	Not stained
* Water	No reaction	light orange No reaction	Sodium and potassium soaps dissolved; calcium and magnesium soaps not dissolved

* Scharlach R and Sudan III solutions are saturated solutions in equal parts 70 per cent alcohol and acetone.

B. Quantitative Determination.—This determination as usually made on the dried feces is very tedious and liable to very erroneous results when applied to pathological feces, due to the changes that occur during drying.

For accurate determination of fat, the twenty-four-hour stool should be collected and thoroughly mixed. A convenient, speedy and accurate method for moist feces is that devised by Saxon. The soaps present are converted into free fatty acids by hydrochloric acid and are extracted with ether, which removes neutral fats, free fatty acids, fatty acids separated from soaps and cholesterol. The ether is removed, the crude fat purified with petroleum ether and the weight of total fat obtained. The fat is then dissolved in benzene and titrated with an alcoholic solution of sodium hydroxide or potassium hydroxide (sodium or potassium alcoholate) and calculated as stearic acid.

1. **Reagents.**—(a) *Sodium (or Potassium) Alcoholate*, 0.1 N.

(b) *Phenolphthalein*, 1 per cent alcoholic solution.

(c) *Benzene*, C_6H_6 .

(d) *Petroleum Ether*; boiling range 30° to 60° C.

(e) *Ether*, anhydrous.

(f) *Hydrochloric Acid*, specific gravity 1.19.

(g) *Ethyl Alcohol*, 95 per cent.

2. **Procedure.**—Thoroughly mix the feces in a mortar or dish and place about 5 gm. (accurately weighed) in a 100-cc. glass-stoppered graduated cylinder. Add 20 cc. of distilled water, 2.5 cc. of concentrated hydrochloric acid, and dilute to 30 cc. with distilled water. Add 20 cc. of ether, stopper and shake vigorously for five minutes. Allow to stand for a few moments, add 20 cc. of 95 per cent ethyl alcohol and again shake for five minutes. Allow to stand until the ether separates out and then blow off the ether (using tubes such as are used in the Warner-Schmidt method for fat in milk) into a clean dry Erlenmeyer flask. Add 5 cc. of ether to the cylinder, flowing it over the stopper; agitate slightly and blow off into the Erlenmeyer flask containing the preceding portion. Repeat this process five times.

Again add 20 cc. of ether to the cylinder and shake five minutes. Allow to separate and blow it off into the Erlenmeyer flask. Again wash five times with ether as before, blowing off each portion into the flask.

Cautiously distil off the ether at low temperature until no odor of alcohol (which was carried over with the ether) can be detected. Add to the flask 30 cc. of petroleum ether, filter through a small plug of cotton (fat-free) in the stem of a small funnel, wash the funnel, flask and cotton four times with 5 cc. portions of petroleum ether and dry the residue at 90° C. Place in a desiccator until cool, then weigh. The difference between this weight and that of the empty flask is the total weight of fat.

After weighing, dissolve the fat in the Erlenmeyer flask in 50 cc. of benzene, heat nearly to boiling and add 2 drops of phenolphthalein solution. Titrate with 0.1 N sodium alcoholate and calculate the weight of the fatty acids as stearic acid.

3. **Calculations.**—The weight of total fat is obtained by subtracting the weight of the empty flask from the weight of the flask and the dried fat. The fatty acids, in milligrams of stearic acid, may be obtained by multiplying the cc. of sodium alcoholate used in the titration by the factor 28.1. The difference between the weight of total fat and the weight of fatty acid is the weight of neutral fat in the sample.

CHAPTER VII

THE SPUTUM

By CARL J. LIND, Jr.

THE specimen should consist of material from the bronchi, rather than saliva or nasal secretion. Thus the best sample is ordinarily an early morning one collected in a clean container with a minimum of mouth contamination. If bronchial secretions cannot be obtained readily, the back of the throat may be irritated with a swab to produce a paroxysmal cough, and the expectorated material caught in a Petri dish. Sputum should be kept cold and examined within a few hours.

BACTERIOLOGY

If the presence of pyogenic bacteria is suspected, make a Gram stained smear and also cultures on blood agar and in infusion broth. For pneumococci, in addition to smear and cultures, a white mouse may be inoculated intraperitoneally and typing attempted with the peritoneal fluid, in case direct typing fails. If tubercle bacilli are anticipated, make an acid fast stained smear and culture and inoculate animals. It is usually difficult to find tubercle bacilli in the bloody sputa of tubercular patients. For the technics in detail, see the chapters on Bacteriology.

Other infections or infestations may cause findings in the sputum. Among these are yeasts and fungi, particularly actinomyces. Some animal parasites which may be identified are *E. histolytica*, *T. echinococcus* (hooklets and scolices), hookworms and roundworms (larvæ), and lung flukes (ova). For further information, see the chapters on Mycology and Parasitology.

MACROSCOPIC EXAMINATION

One may note layering, mucus, pus, color, odor, coagulation, tenacity and quantity. Curschmann's spirals are yellowish-white masses up to 1 or 2 cm. long of twisted threadlike fibers surrounded by mucus. Bronchial casts and broncholiths are uncommon. Dittrich's plugs, from bronchi or tonsils, are friable, yellowish-white masses 1 to 10 mm. diameter, with a putrid odor.

Sometimes sputum is of unusual color. That from workers in coal or iron may be black, that of bakers and millers, white, that of patients with jaundice or pneumonia, green, that of workers with dyes or pigmented chemicals almost any color. The usual variations are red sputa due to blood, and rusty or brownish sputa due to the chronic pulmonary congestion of pneumonia.

MICROSCOPIC EXAMINATION

I. **Unstained Preparations.**—Choose a suspected particle and spread under a cover glass. Small Curschmann's spirals may be seen (asthma?). Elastic fibers have the appearance described in the chapter on Feces. Charcot-Leyden crystals are needle shaped, 20 to 40 microns long. Rarely,

pneumoliths or small calcified bodies are seen in tuberculosis. "Heart-failure-cells" are macrophages 30 to 40 microns in diameter containing faint brown cytoplasmic inclusions that give a positive iron reaction. Similar cells carrying brown-blackish carbon occur in anthracosis. Myelin forms colorless globules, which in larger forms have concentric or irregular spiral lines. The yellow or gray sulfur granules of actinomycosis show a mesh of mycelia when crushed. Non-pathogenic molds and yeasts may be seen. Spores occur as highly refractile spheres.

II. Stained Preparations.—Prepare moderately thin films, air dry and then fix in a flame, or with absolute methyl alcohol, or 1 per cent mercuric chloride solution. For bacteria see the pertinent chapters. For cells, any polychromatic dye is suited, such as Wright's stain. Erythrocytes are normally degenerated and remain only as pigment. The presence of many neutrophiles suggests a pyogenic reaction. Lymphocytes are normally common, but when present in excess they suggest a chronic reaction such as tuberculosis. Eosinophiles are common, fragile, and usually occur in large numbers in acute asthma. A differential eosinophile count of over 25 per cent suggests an allergic state. Epithelial cells are of three forms. Squamous cells are large, flat, polygonal, with a small nucleus, and are derived from the upper respiratory tract. They are especially numerous in acute pharyngitis and laryngitis. Glandular (cylindrical) cells are from the nose, trachea and bronchi. They are usually infrequent and in varying stages of degeneration, although cilia may persist. Alveolar cells are rounded, 20 to 40 microns in diameter, with one or two round nuclei.

A diagnosis of tumor from sputum examination should be made with caution. For this type of study, in addition to direct smears, the sputum should be mixed with saline and the sediment examined both in smears and after fixation and section. Carcinoma is difficult to diagnose unless masses of the tumor tissue are found. Similar caution should be observed in the attempt to interpret inclusion bodies in the cells as indicative of virus infection. The Giemsa stain is generally a good stain for inclusion bodies.

CHEMISTRY

An occasional test for ptyalin (salivary amylase) is the only commonly used chemical procedure. To a few cubic centimeters of saliva in a test tube add a few drops of 1 per cent soluble starch solution and incubate at 38° C. for thirty minutes. If ptyalin is present the starch is digested, and fails to produce a blue color when Gram's iodine solution is added to the incubated mixture.

CHAPTER VIII

TRANSUDATES AND EXUDATES

By CARL J. LIND, JR.

GENERAL

ABNORMAL fluids from body cavities are roughly classified as transudates and exudates. Transudates are considered as mainly filtrates generally due to mechanical causes, while exudates are commonly the result of inflammation.

Specimens should be collected in sterile clean containers. Potassium oxalate may be added as an anticoagulant. The specimen should be

riological

TABLE 8

	Transudates	Exudates
Specific gravity	Well under 1.018	Well over 1.018
Appearance	Watery to slightly yellow, clear to hazy	Cloudy to purulent
Albumin	Well under 2.5 per cent	Well over 2.5 per cent
Microscopic	No bacteria; a few red blood cells and mesothelial cells	Usually bacteria with generally a marked cellular exudate
Spontaneous coagulation . .	None	Frequent

Determine specific gravity with a hydrometer. Albumin may be determined by the chemical methods elsewhere described, or by adding a few drops of 5 per cent acetic acid. On adding this acid, transudates show no reaction to a slight turbidity; exudates immediately form a dense white cloudy precipitate.

MICROSCOPIC EXAMINATION

A total cell count is rarely indicated, but a differential count is of great value. The fluid must be fresh and unfixed. Smears are made from the well mixed specimen, and also from the centrifugalized sediment. Stain these for bacteria and also as a blood film. It is important to have a thin film, rapidly dried, or else the cells may be shrunken and difficult to identify. For microscopic section, the packed sediment may be overlaid with any of the usual fixatives (*e. g.* 10 per cent formalin). After this hardens gently dislodge the plug, block vertically and proceed as for any histological preparation.

The cytological findings are usually not diagnostic. However, they are of great value in suggesting probabilities. Many neutrophils will suggest a pyogenic infection. An excess of lymphocytes occurs in tuberculosis, chronic infections and old effusions. Mesothelial cells are usually very large, with abundant cytoplasm, and one or more round vesicular nuclei. They tend to clump in masses, are variable in size and frequently show degenerative cytoplasmic swelling. These cells predominate in transu-

dates, and when present in large numbers with associated erythrocytes are common in malignancy. They do not show mitosis. It is essential not to mistake these for cancer cells. Eosinophiles are occasionally prominent, sometimes with tissue basophiles, in pleural effusions, especially after pneumothorax. Erythrocytes, frequently undergoing degeneration, are prominent in tuberculosis and malignancy. Malignant cells are hard to recognize except when undergoing mitosis or arranged in clumps maintaining a tissue pattern. Individual glandular tumor cells containing mucus may be identified. These cells are easier to recognize in the microscopic section, and even there, the diagnosis must be made with caution. Crystals are usually a sign of encapsulated or encysted fluid. Cholesterol forms are usually a result of fatty degeneration; hematoidin forms, of old blood.

CHARACTERISTICS OF VARIOUS FLUIDS

I. Pleural.—A red color is generally due to fresh blood, and is ordinarily associated with trauma, tuberculosis, neoplasm, aneurism, cirrhosis of the liver or hemorrhagic states. A brownish color implies the presence of old blood, and suggests a neoplasm but a clear fluid by no means rules out malignancy. Empyema fluid ranges in color from yellowish brown through yellow to gray. A milky appearance may be due to fat from the blood or associated with pneumococcus infections, a

II. Peritoneal.—Ascites is usually associated with neoplasm, cardiac or renal disease, cirrhosis, or inflammations of the peritoneum. A tuberculous ascites is a typical exudate, with elevated specific gravity and albumin, which clots spontaneously. It is usually yellowish, occasionally with a greenish tint, and its cells are mainly lymphocytes, although in the acute phases there may be many neutrophils, with or without erythrocytes. Tubercle bacilli are rarely found on the direct examination. In cirrhosis the cells are few, mainly flat mesothelial forms with occasional lymphocytes. In malignancy, there are usually many cells, mostly mesothelial, and occasional clumps of tumor cells. An opaque ascitic fluid resembling watery milk is either chylous or pseudochylous. The latter is due to degenerating mesothelial cells or to albumin.

III. Pericardial.—A peculiar non-suppurative effusion occurs in cases of renal and cardiac disease. This transudate is alkaline, usually clear, colorless to a yellowish-green tint. Its albumin exceeds 3 per cent and it may clot on standing. It is sometimes turbid due to degeneration of mesothelial cells. Malignancy causes reactions similar to those of the pleura.

IV. Synovial Fluid.—This is normally alkaline, thick, viscid, yellowish, and may appear cloudy due to cellular debris. It contains albumin and a mucin-like substance.

V. Hydrocele Fluid.—The fluid is clear, frequently dark green to yellow. Specific gravity ranges from 1.014 to 1.026, solids about 6 per cent, and it may coagulate spontaneously. It always contains moderate numbers of lymphocytes, and may have cholesterol crystals.

VI. Spermatocoele Fluid.—The fluid is thin, and resembles watery milk. The specific gravity is usually 1.006 to 1.010. Ordinarily spermatozoa may be found.

VII Pancreatic Cyst Fluid.—The fluid is variable. It is usually neutral to alkaline, and contains blood. Reported specific gravity varies from 1.007 to 1.030. Most fluids contain amylase or trypsin, and are free of albumin.

VIII. Hydatid Cyst Fluid.—The fluid is usually clear and colorless, with specific gravity 1.005 to 1.015. There is little albumin. Usually fragments of the organism may be found in the sediment. (See Section on Helminthology.)

CHAPTER IX

THE CEREBROSPINAL FLUID

BY CARL J. LIND, JR.

THE fluid is collected preferably in three sterile, chemically clean test tubes, numbered 1, 2 and 3. The first drops (tube 1) may contain some blood from the puncture. This fluid should be used only for bacteriological procedures. Part of the contents of the last fraction (tube 3) should be removed for cell counts. Tubes 2 and 3 may be used for any indicated procedures. When a canary yellow fluid (xanthochromia) is obtained, it is advisable to use a trace of potassium oxalate in tubes 2 and 3 to prevent clotting. The cell studies and indicated bacteriological procedures should be done at once, but the remaining tests may be delayed several hours if the specimen is refrigerated. Always shake each tube gently before withdrawing a sample.

On cerebrospinal fluid, the usual serological test for syphilis is the Wassermann. For details see Chapter XII.

GROSS AND MICROSCOPIC EXAMINATION

I. *Physical Characteristics*—Normal fluid looks like water, is alkaline

ity. A dull red or brownish color usually is due to old blood in the fluid. Always report the presence of fresh blood. Xanthochromia is associated with a high globulin in a fluid which usually clots spontaneously on standing. Coagulation is abnormal, and generally associated with meningeal reaction. To examine for pellicle formation, set the tube upright and do not disturb it. A pellicle forms on the surface of the fluid, and extends down

formation does not rule out these diseases.

II. *Cytology*.—*Total cell count* is best made with the Fuchs-Rosenthal counting chamber. The ruled area in the chamber covers 16 square mm. and the coverglass distance is 0.2 mm. forming a volume of 3.2 cu. mm. A polychromatic staining solution (such as methyl or gentian violet, 0.2 gm.; glacial acetic acid, 10 cc.; distilled water 90 cc.; well filtered) is drawn to the mark 1 in a regular leucocyte pipet. Then the well mixed spinal fluid is drawn to the mark 11. Shake the pipet well, discard a few drops, place a drop in the counting chamber, wait several minutes for the cells to settle, and then make the count. The exact count per cu. mm. is the number of cells in the chamber divided by 3.2 (chamber volume), multiplied by 11/10 (dilution factor) the number of

If the Fuchs-Rosenthal chamber is not available, the ordinary nemacytometer counting chamber may be used. Here the calculated number of

cells per cu. mm. must be multiplied by 11/10 to compensate for the dilution.

With a very high cell count, the fluid may be diluted with the staining solution to facilitate counting. An elevated count on a bloody fluid is unreliable. A normal count is considered to be under 10 cells per cu. mm.

Differential Cell Count.—Prepare thin smears of centrifugalized spinal fluid sediment. If the cells will not remain on the slide, one may add a little acellular plasma or serum, or better, fix in an albumin fixative. Quick drying in air is satisfactory. The stain may be a weak aqueous methylene blue solution, or Wright's stain diluted with one-third volume of absolute methyl alcohol. A differential count from the Gram stained preparation is not satisfactory, and a differential should not be done in the counting chamber during the total cell count.

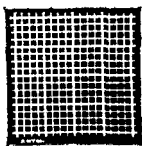


FIG. 5.—Fuchs-Rosenthal counting chamber.

The differential should report the percentages of lymphocytes, neutrophils and endothelial cells. Count at least 100 cells. Normally one sees few cells except lymphocytes.

BACTERIOLOGY

Prepare smears of the sediment from the centrifugalized fluid. Make a Gram stain for organisms. For tubercle bacilli, centrifugalize at high speed for thirty minutes, and stain the sediment by the acid fast method. A coagulum may be stained for bacteria and is frequently positive when the remaining fluid is negative. A pellicle is the best part of the specimen to stain for tubercle bacilli. For cultural and inoculation methods see the chapter on Bacteriology. If a relatively delicate organism such as the meningococcus is suspected, there should be no delay in proceeding with the bacteriological studies.

CHEMICAL EXAMINATION

I. Protein.—The chief
about 30 mg. per 100 cc.
invalidates a determination.

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1. Pandey's Test.—This is a qualitative test. Melt phenol crystals, CP, in a container surrounded by hot water, then make a saturated solution of phenol in water by putting 100 cc. of and adding distilled water up to 1000 cc. days in which it is kept in an incubate

To perform this test, add a large drop of spinal fluid to 1 cc. of the reagent. A bluish white cloud immediately forming around the drop as it swirls in the reagent indicates an abnormal increase in the protein. Normal fluids often show a faint trace by this method. This trace should not be reported. Report the test as negative, moderate increase, or marked increase.

2. Esbach's Test. (Lundeberg.)—This gives a roughly quantitative result. Using a reagent of 10 cc. of trichloroacetic acid in 90 cc. of distilled water, make the test in the same manner as described for urine, except that the following device is recommended for small quantities of spinal fluid. A narrow test tube, or large glass tubing sealed at the bottom and about 5 mm. in diameter, is strapped with adhesive tape to the side of an Esbach albuminometer tube. Spinal fluid is poured in to opposite the U mark and the reagent to the R mark.

... .. : enis and Ayer, modified.)—The turbidity when added to a solution containing the concentration of the protein in the solution. In this test the turbidity of the unknown is compared to the turbidity of a solution containing a known amount of protein. For this test a cloudy or bloody specimen must be centrifugalized to obtain clear fluid, but xanthochromia does not interfere. This is an accurate, relatively simple test.

Make a standard solution of pooled normal serum diluted with 15 per cent sodium chloride solution to contain an exactly determined amount of protein, most conveniently between 20 and 40 mg. per 100 cc. This measured value in mg. per 100 cc. is called "K" in the calculations below. If a few crystals of thymol are added this solution will keep for six months in the refrigerator.

To perform the test, make a mixture of 0.6 cc. of spinal fluid, 0.4 cc. of distilled water and 1.0 cc. of 5 per cent sulfosalicylic acid. Mix this by inversion, but not by violent shaking. Make a second mixture of 1.0 cc. of the standard to stand for at least five minutes. Then compare both in a colorimeter (Dubosq) or photometer (maximum transmission at 400 mμ).

The calculation for the colorimeter is:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{K}{0.6} = \text{mg. protein per 100 cc. in unknown}$$

As long as the proportions are unchanged in the spinal fluid mixture, the volume of the spinal fluid may be increased or decreased without changing the formula. If the turbidity of the spinal fluid mixture is compared with distilled water and at the end of the

... .. values for sugar lie between 40 and 70 mg. per 100 cc. This determination is best done quantitatively by the methods described for blood chemistry. Chlorides, alcohol, and other constituents are best determined similarly.

PRESUMPTIVE TEST FOR TUBERCULOUS MENINGITIS

In tuberculous meningitis the ratio of the precipitate in the spinal fluid on adding mercuric chloride to the precipitate on adding sulfosalicylic acid is frequently altered (Levinson's method). This test is presumptive but not diagnostic.

CHAPTER X

THE BLOOD

By CARL J. LIND, JR.

Over 90 per cent of hospital laboratory procedures involve examination of the blood. Hence one should avoid unnecessarily elaborate or difficult techniques whenever possible. The attempt in this chapter is to indicate simple methods suitable for definitive procedures for over 90 per cent of ordinary work and the latter are procedures of clinical research.

In this country the determination of hemoglobin, red and white cell, and differential counts are the ordinary basic or routine blood studies. When these values vary from normal, or the clinical picture warrants it, the definitive procedures should be performed.

The majority of the methods described here are quantitative and objective. A chapter on blood methods cannot cover completely the vast scope of morphological interpretation. For details of cell structure and clinical significance readers are referred to the many standard texts on pathology, histology and hematology.

GENERAL METHODS

I. Specimens.—1. Skin Puncture.—Skin puncture of the finger tip, the lobe of the ear, or in infants of the ball of the heel, is the usual source of blood. In any case the site is gently massaged or placed in warm water to promote active congestion. This provides a standard specimen. Parts showing pallor, cyanosis, edema or inflammation contain abnormal blood. Too brisk rubbing will increase the clotting factors and alter the sample. The part should be cleaned, then defatted with alcohol which is allowed to dry.

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With alcohol commonly used. After the vein is entered, release the tourniquet, draw

the specimen, compress the site with a sterile pledget and withdraw the needle. Then remove the needle from the syringe and gently express the blood into the proper container. It is particularly important to release the tourniquet before drawing blood for a sedimentation rate or hematocrit determination.

The same syringe may be used repeatedly if it is well rinsed after use with a sterile saline solution. Technicians should observe care not to contaminate the fresh sterile needle which must replace the used needle. The saline should be changed frequently to avoid hemolysis. If hemolysis occurs, obtain another sample.

Other venepuncture sites are the forearm, the hand, the popliteal veins of females, and the longitudinal sinus or the external jugular vein in infants. If the vein is very small, a needle of less than 19- or 20-gauge may be used. Hematomas are generally due to multiple punctures in the same region or to penetration of the deep wall of the vein. If bleeding into the tissues begins, apply pressure and choose another site.

3. Anticoagulants.—The best anticoagulant is heparin. To use, add a pinch of the powder to the specimen tube. The amount is usually very small, roughly 0.2 mg. per cc. of blood, and can be judged best by trial. The drug may be dissolved in saline, and the solution dried in the tube. Mixing must be thorough. Heparin has no effect on the size of the cells. Some observers think it causes a decrease in the number of platelets but this is doubtful.

Another good anticoagulant that is more suited for routine work is 6 mg. of ammonium oxalate and 4 mg. of potassium oxalate per 5 cc. of blood (Heller and Paul). To prepare a stock solution, add 12 gm. and 8 gm. respectively, of the dry chemicals to 1000 cc. of distilled water. If 0.5 cc. of this is added to the collection tube and allowed to dry on the wall, the tube is then prepared for 5 cc. of blood. This anticoagulant has no effect on the size of the cells. A frequently used anticoagulant is a drop of 20 per cent sodium or potassium oxalate per 5 cc. of blood, although this, like others in common use, causes changes in cell volume and cell characteristics.

Whenever venepuncture is done, the specimen bottle should be tightly corked if the blood is not used at once. This will prevent evaporation of the plasma. Sedimentation begins at once, so that the blood should always be thoroughly mixed before removing a sample. In general, keep the bottle stored in the refrigerator and use as soon as possible. In the absence of contamination, platelets degenerate in one to several hours, granulocytes in six to twenty-four hours, lymphocytes alter in a day or so, and erythrocytes change slowly. Contamination accelerates these changes.

II. Routine Equipment.—**1. Syringes and Needles.**—To prepare a syringe for sterilization, wrap the plunger and barrel separately in gauze, and then wrap both in heavy paper, and secure with twine. To prepare a needle for use, replace stylet with loop at point for protection of the edge and slip the needle, point down, into a Wassermann tube, plug with cotton, and sterilize. Both needles and syringes can be sterilized alike. An autoclave at 121° C. (15 pounds steam pressure) for fifteen minutes, or dry heat at 170° C. for two hours will effect sterilization.

Always wash out a used syringe before the blood clots in it. If the syringe is to be stored, remove the plunger. If the plunger freezes in the

barrel of a syringe, one may put the cooled syringe in warm water to expand the barrel, and then try to pull apart. If this fails, fill another syringe with water, put on a needle which can be inserted into the stem of the frozen piece, and force this water against the head of the frozen plunger. The freeze may be loosened by injecting hydrogen peroxide solution. Another method is to wind the barrel with rubber tubing, and the head of the plunger with similar tubing but in an opposite direction, and then twist. The glass should be well covered for protection in case of breakage.

Needles should be rinsed immediately after use, or dropped in water. As soon as convenient, clean them thoroughly with cold water, and dry by forcing alcohol followed by ether through the bore. Wet needles will rust. Sharpening can be done on the finest grade of emery cloth spread on a flat surface. This should be followed with a few strokes on a fine blue water stone. The pain of venepuncture is related more to dullness than to the size of the needle.

2. **Glassware.**—All glassware must be scrupulously clean, without fat or oil film. New slides should be washed in hot soapy water, rinsed very well, and placed in a beaker of 95 per cent alcohol for storage. For use they are polished with chamois or a lint-free cloth, such as old linen, and flamed. They may then be stored in a box with a clean piece of paper between each slide. Dirty or used slides should be salvaged. Boil them in 5 per cent sodium bicarbonate solution, then scrub with soap and water and place in sodium dichromate-sulfuric acid cleaning solution for twelve hours, and finally wash as for new slides. Cover slips can be treated similarly except that they must not be flamed. A final rinse in ether speeds drying.

Counting chambers are cleaned with soap and water, rinsed in distilled water, and air dried. If a chamber is needed at once, blot it dry with lens paper. Alcohol and ether may be used with care to remove oil from a counting chamber, but xylene and other cement solvents must never be used on cemented chambers.

New hemocytometer pipets should be cleaned before use. Using suction, draw water through them, followed by alcohol, ether and air. The bead in the bulb should shake freely in a clean pipet. If a pipet is plugged, the capillary bore may be cleaned with a horsehair and the tube soaked twelve to twenty-four hours in dilute nitric acid and then cleaned as above.

III. **Preparation of Blood Films.**—1. **Dry Films.**—Place a drop of blood on one end of a clean slide held on a flat surface. Hold the other end of the slide firmly. Choose another slide with a regular unchipped edge at the end. Place this edge transversely across the center of the flat slide and draw the edge up to the drop of blood at angle of about 30 degrees, so that the blood by capillarity fills the acute angle between the flat slide and the top slide. Then push the top slide with a firm steady motion toward the opposite end of the bottom slide, maintaining the same angle. Thus the blood is drawn in a thin film over the bottom slide. The slower the movement and the greater the angle between the slides, the thicker the film will be. The film should be dried at once by waving it in the air. Rapid drying causes the cells to spread well and to present more surface for examination. Ordinarily heat should not be used, although gentle heat may be necessary in very humid climates. In a dry atmosphere an electric charge may

accumulate on the glass and cause an unequal flow of blood. This can be discharged by passing the slide through a flame before use.

Films may be prepared on cover slips. The size of choice is 22 x 22 mm., No. 2 thickness. Pick up two clean slips by their edges. Let a drop of blood about 2 mm. in diameter flow on the center of one. Then place the other slip flat over the drop so that all the slip corners form an eight-cornered star. Capillarity spreads the drop of blood. Just before spreading is complete, separate the slips with a sliding motion. Do not lift slips apart. Do not let the blood clot. Then air dry the film as above.

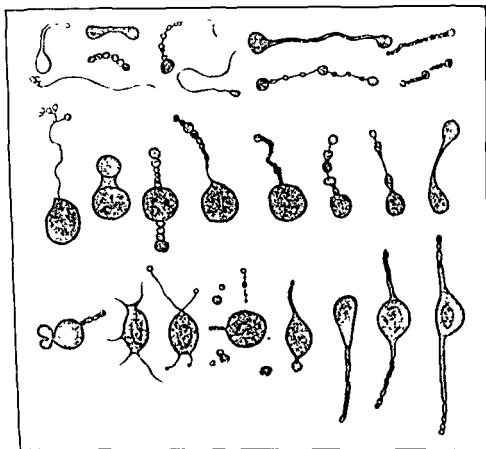


FIG. 7.—Altered red cells. The chain, dumb-bell, droplet, and filament phenomena in fowl's blood and human blood as seen by the dark-field method. *Not drawn to scale.* (In part after Nuttall and Graham-Smith, from Andrew Balfour's *Fallacies and Puzzles in Blood Examination*, courtesy of Baillière, Tindall & Cox.)

These preparations are variously called—slides, slips, smears, spreads, streaks and films. The blood is best obtained from a skin puncture. Venepuncture blood is not advisable, as the normal values differ slightly and sedimentation will have begun. The cover slip method is more accurate for a differential count particularly if all the leukocytes are counted. Another advantage of the cover slips is that they can be stained by immersion. However either method can give proper films, in which there is an even spread, thin enough so that the red cells lie flat and abutting, and do not overlap. The secret of good film technic lies in clean glassware, a thin film, and rapid drying.

Films should ordinarily be stained soon after preparation. After a

few days a film loses its staining characteristics. An unfixed film may be eaten by insects. If a delay is necessary, fix the film for three to five minutes with absolute methyl alcohol, or coat with melted paraffin which may be removed later with xylene.

Films may be marked for identification with a glass pencil, a wax pencil, or most conveniently by writing over the edge of the blood film with a lead pencil.

A valuable accessory procedure for morphological study in a patient with severe anemia or abnormal plasma is to obtain blood by venesection, mix with anticoagulant, centrifugalize gently, remove most of the supernatant plasma and replace it with normal compatible plasma equal in volume to the cells. If only leukocytes are desired, as in leukopenia, make films from the gray buffy coat above the red cells. Artefacts are frequent, especially after vigorous centrifugation, so that this should be considered a supplemental procedure only.

2. Wet Films.—These preparations provide a view of the whole blood, in its living state. To prepare the film, touch a very small drop of blood from a fresh skin puncture to a cover slip, which is immediately placed blood side down on a slide. The glass must be clean, the procedure gentle. After the blood spreads beneath the cover slip in a thin film, seal the edges with petrolatum. In a satisfactory preparation the red cells are separated from each other and there are no rouleaux.

By this technic, one may observe the shape and size of the erythrocytes, the mobility and activity of the leukocytes, fibrin formation, and certain parasites (malarial pigment, moving parasites). Dark-field illumination gives added information.

IV. Stains and Staining.—There are many methods of staining blood films. The customary ones use a polychrome methylene blue-eosin stain. Fixation is usually done by the methyl alcohol in the stain or by previous treatment with absolute methyl alcohol. Since polychromation is delicately related to the pH, buffers are frequently used instead of tap water of unstable composition or distilled water which is more or less acid due to dissolved carbon dioxide. For any given region, the water can be tried and corrected to suit the examiner.

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In a proper stain by either method the red cells are a buff color, the granules of the neutrophils lilac, of the eosinophils bright red, and of the basophils deep blue, and the platelets are purplish blue with a distinct architecture. If the solution is too acid, the erythrocytes are a bright red, and the leukocyte nuclei are a pale blue to colorless. If the solution is too basic the red cells are a slate blue and there is little differentiation.

1. Buffers.—(a) pH 6.4 (best for Grüber stains).

Sodium dibasic phosphate (anhydrous)	2.56 gm.
Potassium monobasic phosphate	6.63 gm.
Distilled water	q. s. ad 1000.00 cc.

(b) pH 6.6 (best for American stains).

Sodium dibasic phosphate (anhydrous)	3.80 gm.
Potassium monobasic phosphate	5.47 gm.
Distilled water	q. s. ad 1000.00 cc.

If the anhydrous dibasic sodium phosphate is not available, the crystalline form with two waters of crystallization ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) may be used in the ratio of 5 parts to 4 of the anhydrous form. The two water crystals in turn can be made from the twelve water crystals by spreading the latter on a glass plate in a desiccator for two weeks.

The weights should be accurate, and volumes must be corrected to standard temperature. By varying the proportions of the chemicals, the buffer can be made more acid or basic so as to give the colors of a proper stain described above.

2. **Wright's Stain.**—The staining powder is methylene blue polychromated with sodium bicarbonate and heat to which eosin is added.

(a) *Preparation of Stock Solution.*—

Wright's stain powder	0.3 gm.
Glycerol, CP	3.0 cc.
Methyl alcohol (absolute, acetone-free)	97.0 cc.

Put the powder in a dry mortar, grind with a pestle, add the glycerol and grind together thoroughly. Add the methyl alcohol and mix. Allow to stand overnight in a tightly stoppered flask, then filter and let the filtrate stand for about a week before using. Some hematologists buffer the

alcohol. This is let stand and then titrated against a neutral indicator and corrected with more phosphate.

(b) *Procedure.*—Cover the dry film with 10 drops of Wright's stock stain, for one minute. This provides fixation. Enough stain must be used of distilled water to make a thick suspension. Let stand for 15 minutes. The stain should appear brick-red. Wash with distilled water, off, and no precipitate should remain. Dry by placing on a slide.

3. **Giemsa Stain.**—The powder consists of Azur II-cosin and Azur II in the ratio 15:4.

(a) *Preparation of Stock Solution.*—

Giemsa powder	0.5 gm.
Glycerol, CP	33.0 cc.
Methyl alcohol (absolute, acetone-free)	33.0 cc.

Dissolve the powder in the glycerol. After several hours add the alcohol. The stain improves with age.

(b) *Procedure.*—Add 1 cc. of stock solution to 10 cc. of distilled water or buffer solution. Stain the previously fixed slide fifteen to thirty minutes, exact time determined by trial. Wash with distilled water and dry.

Since the Giemsa stain is poor for granules and cytoplasm it is usually preceded by a Wright's stain or the May-Gruenwald stain. The latter combination is called by some the Pappenheim panoptic stain.

The technic for staining thick blood films for malaria or other parasites is described in the Chapter on Protozoology.

4. **May-Gruenwald Stain (Jenner's Stain).**—The powder is Jenner's cosinate of methylene blue.

(a) *Preparation of Stock Solution.*—Cautiously heat 100 cc. of absolute, acetone-free methyl alcohol to 50° C. Slowly add 0.5 gm. of May-Gruen-

wald powder. Let stand for twenty-four hours. Filter and store in a dark bottle.

(b) *Procedure*.—Flood the unfixed slide three to five minutes, rinse with water and dry. This stains granules well.

Stains vary from batch to batch. This occurs less with certified stains. Solutions are usually more permanent in pyrex glass than in ordinary alkali glass. Keep bottles of stain well corked to prevent evaporation. To avoid sediment on a film, a pressure blowing bottle may be used for the final rinse, or the film may be stained on edge or face down. A poorly stained or faded film may be decolorized with absolute methyl alcohol and restained.

Permanent mounts may be prepared with a slide and cover slip. An important point here is the pH of the mounting medium. The artificial resins (Clarite) are much more stable in their reaction than balsam. Such mounts should be kept out of strong light to decrease the fading.

5. *Peroxidase Stain* (Sato and Sekiya).—The myeloid leukocytes contain an oxidizing ferment which in the fresh unfixed film causes benzidine to be oxidized and deposited as cytoplasmic granules.

(a) *Materials*.—Solution A is 0.5 per cent copper sulfate solution. Solution B is 0.2 gm. of benzidine in 200 cc. of distilled water to make a saturated solution at room temperature. This is filtered and 4 drops of fresh 3 per cent hydrogen peroxide solution are added. This solution should be kept in the dark when not in use. If mixing solutions A and B does not give a blue color, solution B must be made again.

(b) *Procedure*.—Place solution A on a fresh dry blood film for thirty to sixty seconds. Pour or wash off gently. Cover slide with solution B for two minutes, then wash and dry. A counterstain (1 per cent aqueous safranin) may be used.

This stains the myeloid cytoplasm blue, with peroxidase positive granules bluish green and eosinophil and basophil granules strongly blue. Monocytes have variable faint small blue granules. Lymphoid cells will not show blue or green granules.

THE WHOLE BLOOD

The blood is about one-twelfth of the body weight, or 5 or 6 liters in volume for the average adult.

1. *Specific Gravity*.—This and the packed cell volume (hematocrit) have received attention as a measure of hemoconcentration, particularly in shock. Normal values range from 1.048 to 1.066. It may be measured by direct weighing, by measuring the specific gravity of a mixture of fluids (chloroform and benzene) in which a blood drop hangs suspended, and by use of falling drop methods in which the rate of fall of the blood in a known fluid is compared with rate of fall of a known standard. To date the results have been somewhat equivocal, although the principle promises much. Detailed instructions for this procedure ordinarily accompany the special apparatus used.

A newer and far more accurate method for measuring specific gravities of whole blood and plasma is the copper sulfate method of Phillips and Van Slyke recently developed. By the use of line charts plasma proteins,

hemoglobin and hematocrit may be calculated from plasma and whole blood gravities.*

II. Blood Viscosity.—This is a common determination in European clinics. In a viscosimeter (Hess) the rate of flow of blood through a capillary tube is compared with that of distilled water. Normal adult blood is 3.5 to 5.4 times more viscous than water. Instructions ordinarily accompany the viscosimeter.

III. Hematocrit (Packed Cell Volume).—Many consider the hematocrit value second in importance only to the blood film. While, strictly speaking, the hematocrit is a tube in which whole blood is centrifugalized to separate cells and plasma, by usage, and in this chapter, it also will mean the percentage of whole blood volume which is cells. The present methods are not exact, but the standardized test (Wintrobe) is of great value, and its results are reproducible.

1. Procedure.—Place exactly 1 cc. of blood treated with an anticoagulant that does not affect cell size or shape in the Wintrobe hematocrit tube. This is a flat-bottomed tube in which 1 cc. of blood forms a column 10 cm. high. The tube wall is marked in mm., thus giving a direct reading of per cent of the 10 cm. height. Roughly equivalent tubes may be improvised from stock laboratory tubing having an inner bore of 4 mm. diameter. The blood is placed in the tube by means of a capillary pipet, avoiding bubbles. Then centrifugalize at 3000 r.p.m. for thirty minutes at a head radius of 9 cm. In the absence of data on centrifuge speed, that speed should be chosen which by trial on normal blood causes slight packing after twenty minutes, but none after thirty minutes. Evaporation of the plasma may be disregarded if the tube was exactly filled, or the original volume noted and a correction factor determined.

2. Results.—(a) *Volume of packed red cells*—normal adult male 47 per cent \pm 7; normal adult female 42 per cent \pm 5. (b) *Buffy coat*—a pale reddish gray layer of leukocytes and platelets overlying the red cells. Each 0.1 mm. thickness is roughly equivalent to 1000 leukocytes per c.mm.

A graduated centrifuge tube may be used, although the results are not standardized. Using the same centrifuge factors, the values obtained average about 5 per cent lower than with the Wintrobe tube.

The hematocrit tubes are best cleaned with flowing water and suction. If left inverted they dry. They may be dried rapidly with alcohol and ether. They require occasional cleaning with strong acid solution.

IV. Sedimentation Rate.—The cells of normal blood settle out slowly on standing. The rate is frequently increased in disease, and appears to be associated with the rate of tissue destruction in the body. Its main value is as an index of progress of a disease. There are many methods. All require an anticoagulant which does not alter the blood cells. The tubes must be exactly vertical. The temperature should be between 20° and 25° C. The specimen should be tested within two hours.

1. Cutler Tube Method.—The tube has a capacity of 1 cc. and is marked in 50 divisions of 1 mm. each, with zero at the top. Into a 2-cc. syringe, draw 0.1 cc. of 3 per cent sodium citrate solution. Then add 0.9 cc. of venous blood. Mix well and pour into the Cutler tube. The height of the column of cells is read every five minutes for an hour and the results

* Published as a monograph at The Hospital of the Rockefeller Institute for Medical Research.

plotted. The normal rate of fall is practically constant, reaching in one hour less than 8 mm. for men, less than 10 mm. for women.

The method may be modified, using tubes improvised from 75 x 10 mm. stock test tubes, selected so that 2 cc. of liquid forms a column 50 mm. high. Etch the tube at this point. Put exactly 0.2 cc. of 3 per cent sodium citrate solution in the tube. Then fill to the etched mark with blood and mix well. Set the tubes vertically and measure the heights as above. Normal rates are slightly higher by this method.

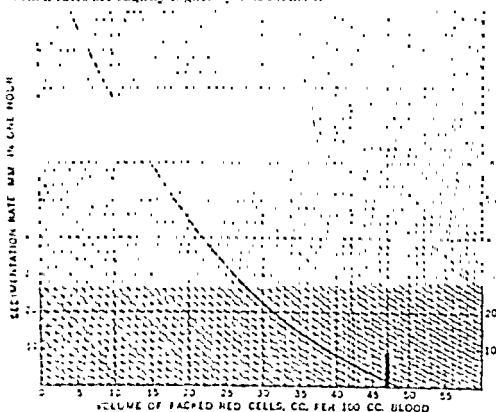


FIG. 8. Chart for correction of sedimentation rate according to hematocrit values. (Wintrobe and Lenzlinger, *Ann. Jour. Med. Sci.*)

2. Wintrobe Tube Method.—The tube is filled to the 10-cm. mark, exactly as for the hematocrit. It is then let stand for one hour, when the uncorrected sedimentation rate is read. Then a hematocrit is determined for the same specimen and the corrected sedimentation rate determined from the graph (Fig. 8), which deducts the sedimentation due to anemia.

To use the graph, follow the horizontal line which represents the uncorrected rate in one hour until it intersects the line which represents the actual hematocrit. Follow the nearest curved line until it intersects the heavy line at hematocrit 47 for men (or 42 for women). From this point of intersection the nearest horizontal line is the corrected sedimentation rate. By this method the normal for men is up to 9 mm.; for women up to 10 mm. It is wise to report both the corrected and uncorrected rates and the hematocrit. This method of method is approximate.

ELLIDING AND CLOTTING

A commonly accepted view of the clotting mechanism is that prothrombin gives rise to thrombin, which in turn activates fibrinogen to

produce fibrin, the basis of the clot. Fibrinogen and prothrombin are normal blood proteins. The change of prothrombin to thrombin is ordinarily induced by thromboplastin from tissue juices or blood platelets. Calcium must be present. This double enzyme reaction is:

Step I. Prothrombin + (calcium and thromboplastin) = Thrombin

Step II. Fibrinogen + (thrombin) = Fibrin

The chemical anticoagulants prevent clotting by removing calcium. Heparin prevents it possibly by combining with the prothrombin, as a so-called antithrombin.

I. Clotting Time.—1. **Slide Method.**—From a fresh, freely bleeding skin puncture, place a few drops of blood on a slide. Pass a needle slowly through the drop every thirty seconds. When the needle point picks up a fine thread of fibrin, the elapsed time from the skin puncture is the coagulation time. Normal time is two to eight minutes. Skin puncture methods are not very sensitive or accurate because tissue juices mix with the blood, but the method is suitable for routine work.

2. **Venepuncture Method (Lee and White).**—Thoroughly rinse a glass syringe and its needle with normal saline. From a venepuncture, using a minimum of suction, gently and rapidly withdraw blood. Place 1 cc. in each of several clean, saline rinsed Wassermann tubes. Tip one tube at intervals, until it can be inverted without losing the clot. Then the same end-point is noted in a previously unmanipulated tube. The time is from the latter point to the venepuncture. Normal time is six to fifteen minutes. Always perform a control test with normal blood. The ideal temperature for the test is 75° F., but a room temperature from 65° to 90° F. is satisfactory. This is a very reliable test.

II. Clot Retraction.—Place about 3 cc. of fresh blood in a clean test tube. Inspect at one hour and again after eighteen to twenty-four hours. Most technics require that the tube be kept in a 37° C. incubator, but this is not necessary.

Normally some clot retraction and separated serum are noted after one hour, and after eighteen hours the process is marked. A normal clot is tough and elastic. Should the clot adhere to the tube wall it should be loosened to permit retraction. An abnormal clot fails to retract and is soft and friable. Poor retraction occurs with low platelet counts.

III. Prothrombin Time.—If the other clotting factors are normal, it is assumed that clotting time depends directly on the amount of thrombin activity. It is further assumed that prothrombin activity can be measured by its ability to form thrombin. These assumptions are fundamental in Quick's basic prothrombin method. In this test an excess of tissue extract (thromboplastin) is added to fresh blood to initiate thrombin formation, and the time for clot formation is considered a direct index of effective prothrombin concentration. This method is practical and easy, even though it does not allow for variations in the prothrombin conversion rate, or the thrombin-fibrinogen reaction time, or for deficiency of fibrinogen or excess of antithrombin.

1. **Micro Prothrombin Time (Lawson's Variation).**—Place 20 c.mm. of thromboplastin extract (volume of Sahli pipet) in the central hollow depression of a clean hanging drop slide. Make a deep skin puncture which bleeds freely. Discard the first drop. Then take 20 c.mm. of

blood in a dry clean pipet and add to the thromboplastin extract, blowing with the pipet to mix. The measured time is from the mixture to the point when the material first gels and moves as a whole rather than flows. Just prior to this test run a control procedure with a normal person. The control time should be between fifteen and fifty seconds. If the control time is shorter, dilute the extract with normal saline to slow the reaction; if longer, the extract is unsatisfactory and should be discarded. Always report both control and patient prothrombin time.

2. Thromboplastin Extract.—Commercial thromboplastin, procured in ampules, should be diluted according to accompanying directions. This aqueous extract remains potent for about thirty-six hours if kept in the coldest part of the refrigerator.

An excellent extract can be prepared in the laboratory by Quick's method, as modified by Irving. Remove the meninges and vessels from the surface of a fresh rabbit brain. Macerate the brain under acetone. Extract three times with acetone, saving the solid material. Dry this in an incubator at 37° C. overnight. This powder may be sealed in a bottle and stored indefinitely in a freezing chamber. To prepare the extract, mix 0.3 gm. of the powder with 4.9 cc. of 0.85 per cent sodium chloride solution and 0.1 cc. of 1.34 per cent sodium oxalate solution. Shake this well and heat at 45° C. in a water bath for ten minutes. Then centrifugalize for three minutes and test the supernatant fluid for activity. This fluid extract keeps well up to nine months if stored in the coldest part of a refrigerator. In routine use warm the preparation in a water bath at 37° C. for ten minutes, or simpler, hold it in the hand until warm, before withdrawing the required amount.

IV. Bleeding Time.—**1. Duke's Method.**—Clean the ear lobe, and make a fairly deep skin puncture wound. Blot with filter paper every thirty seconds, but be sure not to touch the skin. The size of the wound should be such that the blot after the first thirty seconds is 1 to 2 cm. diameter. The use of the finger as a bleeding site is not as dependable. The normal time from the incision to cessation of bleeding is one to three minutes.

2. Ivy's Method.—Inflate a blood pressure cuff around the arm to a pressure of 40 mm. of mercury. Make a lancet puncture 2.5 mm. deep on the inner side of the forearm just below the elbow. Blot and time as in Duke's method. The normal time by this method is two to four minutes, and this test is more accurate.

THE CELLULAR COMPONENTS

I. Red Cell Count.—**1. Materials.**—(a) The Thoma diluting pipet is used to take the specimen from a skin puncture. The bead in the bulb is often red. There are graduations along the capillary bore. The fifth from the tip is marked 0.5, the tenth 1; above the bulb is a line marked 101. Blood is drawn to the 0.5 mark and diluting fluid is added to reach the 101 mark, making a dilution of 1:200.

(b) The improved Neubauer ruled counting chamber is a thick glass slide with two central platforms. On the surface of each platform are engraved rulings. The side platforms, on which a special thick flat cover glass rests, are exactly 0.1 mm. higher than the central platforms. With cover slip in place, there is a space 0.1 mm. deep over the ruled area. The

ruled areas have a surface 3×3 mm., or 9 square mm. The four large corner squares outside the double ruled lines are each divided into 16 smaller squares. The central square is divided by double lines into 25 small squares, each of which contains 16 very small squares, making 400 very small squares in all. Thus each very small square is $1/400$ sq. mm.

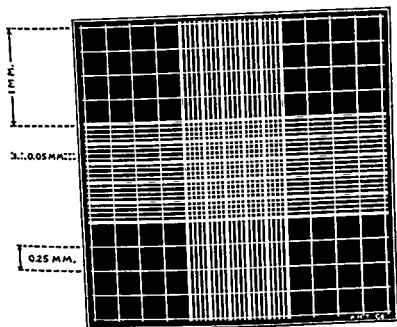


FIG. 9.—Entire area of new Neubauer counting chamber

(c) *Diluting Fluid (Hayem's solution).—*

Mercuric chloride	0.5 gm.
Sodium chloride	1.0 gm.
Sodium sulfate	5.0 gm.
Distilled water	200.0 cc.

2. **Procedure.**—Fill the pipet exactly to the 0.5 mark with blood. Remove the excess on the tip. Add diluting fluid to mark 101. Kink the rubber tube at the end of the pipet against the middle finger and place the tip against the thumb, and shake in a figure-of-eight motion for two minutes to give a good mixture.

in place on the counting

the tip of the pipet to the

fluid to flow under the cover glass

clean the chamber and repeat. When satisfactory, allow the cells to settle two minutes and examine with the high dry power. Count the number of cells in 5 of the 25 small squares comprising the central sq. mm. of the ruled scale. Count cells touching or crossing the upper and right sides of these small squares, but not the cells touching or crossing the lower or left sides. To the total number of these cells add 4 zeros to get the red cell count per c.mm. of blood.

This factor of 10,000 is arrived at as follows:

All cells were counted in 5 small squares, each made up of 16 of the very small squares. Therefore, 80 very small squares, each $1/400$ sq. mm. in area, were counted, or a total of $1/5$ sq. mm. The chamber is 0.1 mm. deep, so the entire volume of diluted blood counted is $1/50$ c.mm. To

convert to 1 c.mm. volume it is necessary to multiply the count obtained by 50.

The dilution is 1:200 and to correct for this the result must be multiplied by 200.

The combined factor is, therefore, 50×200 or 10,000.

The maximum variation in the 5 squares counted should not exceed 15 per cent. If it does, the chamber was dirty, or inaccurate, or there was improper mixing. For accurate work one should either procure certified instruments or calibrate some of his own. Commercial pipets vary as much as 10 per cent in accuracy. They may be corrected gravimetrically on the analytical balance, using colored water. The weight of the dry pipet the weight filled to 0.5 mark and the weight filled to 101 mark provide the necessary data. The counting chambers are usually more accurate. Their grids may be checked with an ocular micrometer, and their cover slip height with the depth of focus micrometer found on most microscope fine adjustment knobs.

II. Hemoglobin.—Hemoglobin is an unstable protein containing 0.0335 per cent of ferrous iron. It is normally present in the blood as reduced

It may be oxidized to a ferric form,
per cent of the total normally, and up
Hemoglobin has two hundred and ten

times more affinity for carbon
with carbon monoxide to form
smokers, about 1 to 3 per cent of hemoglobin is always in this form, and in
cases of poisoning this fraction may rise up to 50 per cent. Carboxyhemoglobin and methemoglobin do not carry oxygen. Certain drugs (acetphenetidin, sulfonamides) may cause the formation of methemoglobin, which may exceed 10 per cent, and sulfhemoglobin which is usually under 10 per cent. In certain conditions (sulfonamides, blackwater fever) hemoglobin in the plasma forms methemalbumin.

In view of the above, it is apparent that various hemoglobinometric methods measure different things, and there is no general clinical agreement as to the potential oxygen cap. These figures are very close.

Hemoglobin determination by crude tests may be reported equally well as per-cent of normal or as grams per 100 cc. of blood. When determined by the more definitive methods, it is best reported by weight. In any event, the method of determination should be clearly understood by all concerned with the test.

For ordinary laboratory work, hemoglobinometers are best calibrated using normal blood at various dilutions. The hemoglobin of the normal blood should be calculated from the iron content of the blood as determined by the method of Wong, page 218.

The following methods are roughly in order of increasing accuracy, and are chosen because they can be used in the average laboratory.

1. Tallqvist Method.—In this test, fresh undiluted blood is blotted on absorbent paper and immediately matched against lithographed colored standards marked 10 to 100 per cent. It is based on 15.8 gm. of hemoglobin per 100 cc. of blood as normal. With its usual variations in paper and color scale, it is very inaccurate in the anemias, and only moderately

accurate with levels close to normal. The great value of this method is its simplicity for use as a screening test in routine work.

2. Sahli Method.—This, like the other acid hematin methods, is much better than the Tallqvist procedure. The apparatus consists of a standard (a sealed tube containing an acid hematin suspension; in older sets a brown colored glass rod), a graduated test tube of the same diameter, a 20 c.mm. blood pipet, and a black rack in which the tubes fit against a white ground glass background. In the procedure, place 0.1 N hydrochloric acid in the graduated tube up to the mark 10. Add 20 c.mm. of blood and rinse the pipet well. Let this stand ten minutes. Then place the graduated tube in the rack and add the diluting fluid drop by drop, with careful mixing. When the colors match, read grams and per cent directly from the tube wall at the upper level of the hemoglobin solution. In the Sahli method 17.3 gm. of hemoglobin per 100 cc. is considered as 100 per cent.

Many other acid hematin methods are in use. The Newcomer method compares the acid hematin solution against a brown glass disc in a colorimeter of Duboscq type (100 per cent considered 16.92 gm.). Using the Hellige-Wintrobe apparatus, blood is diluted 1:100 with decinormal hydrochloric acid in a red cell pipet. This fluid is compared in a cell with a calibrated wedge of brown glass. In the Haden-Hausser instrument the dilution is similar but comparison is against a series of brown glass standards. Objections are that brown is hard to match, that brown standards are variable in their manufacture and deteriorate, that substances in the plasma may add color, and that the color of acid hematin gradually increases in intensity. After ten minutes, only 90 to 95 per cent of the final color is obtained.

3. Alkaline Hematin Methods.—Decinormal sodium hydroxide may be used to dilute whole blood 1:100. The color is permanent but hard to match except photometrically, using a green filter (Wratten 74) and calibrating against an independent standard (*e. g.*, O_2 capacity). A photoelectric cell may be used. If the solution is held in boiling water for five minutes all hemoglobin compounds, including sulfhemoglobin, are turned into alkaline hematin. This is the principle of the Sheard-Sanford photometer.

4. Carboxyhemoglobin Method (Haldane method).—The color is good and carboxyhemoglobin is one of the most stable of known hemoglobin compounds. This method measures hemoglobin, oxyhemoglobin and carboxyhemoglobin of blood. If blood is first reduced with sodium thiocyanate, it also measures methemoglobin. Twenty c.mm. of blood are treated with carbon monoxide (coal gas) and diluted with a solution of 0.1 cc. of strong ammonia water in 100 cc. of water until the color matches the standard solution. Standard tubes are prepared as follows (Donaldson, Harding, Wright): Take 2 cc. of fresh fasting normal blood. Dilute 1:100 with distilled water. Place in a glass-stoppered 500-cc. bottle and gas well with carbon monoxide from the reaction of sulfuric acid with formic acid. Gas again in a few hours to remove other gases. Stopper tightly and refrigerate for two to three weeks. A precipitate will form. Take 2 cc. of the clear supernatant fluid, again gas thoroughly and seal immediately. These standards should last for several years. The colors are easier to match visually than those of other hemoglobin compounds, and spectro-

photometrically (at 540 $m\mu$) there is high reproducibility of results. The standard is calibrated by an oxygen capacity determination done at the same time.

5. Cyanmethemoglobin Method (Stadie).—Hemoglobin, oxyhemoglobin, carboxyhemoglobin and methemoglobin are all transformed to cyanmethemoglobin. Sunderman's modification is relatively accurate and simple. Place 1 cc. of citrated blood in a 100-cc. flask. Add water to hemolyze. Add about 5 cc. of a solution of 0.3 gm. of potassium ferricyanide and 0.1 gm. potassium cyanide dissolved in 100 cc. of water.

Use a Duboseq colorimeter with a wavelength of 530 $m\mu$. Adjust illum-

ination to an even field. Place the solution in one cup. Then match against a standard that decreases light (*e. g.*, monel metal mesh, screening, dark glass disc). The standard must have been previously calibrated against a cyanmethemoglobin solution whose hemoglobin equivalent is known by a concurrent oxygen capacity determination. This method is of course far simpler with spectrophotometric methods. *Caution must be observed in handling the cyanide.*

III. Reticulocyte Count.—This is a supravital staining technic which indicates erythrocyte regeneration. A clean slide is smeared with a few drops of 1 per cent alcoholic solution of brilliant cresyl blue, and then dried. A wet film preparation of fresh blood is made and let stand for ten minutes. Under oil, 1000 cells are counted and those containing bluish strands of reticulum noted. Thinner preparations are easier to count.

This is probably mainly an index of cell shape, the more spherical forms hemolyzing first.

Align two series of 15 test tubes or Kahn tubes, chemically clean. Number each series 1 to 15. Prepare a 1 per cent solution of sodium chloride in distilled water. The first tube of the set of 15 receives 0.6 cc. of the 1 per cent saline, and the others amounts increasing by 0.04 cc. over the amount in the preceding tube; thus, the last receives 1.16 cc. Distilled water is added to make the volume in each tube 2 cc., which gives a series of salt concentrations ranging from 0.30 to 0.58 per cent by increments of 0.02 per cent. Obtain blood from the patient by venepuncture and immediately add 1 drop to each tube in one series. Repeat for the other series of tubes with blood from a normal control. Shake all tubes and let stand for two hours at room temperature. To read the test the highest concentration of saline showing any red or pink in the supernatant is recorded as beginning hemolysis; the highest concentration showing no cells is recorded as complete hemolysis. The concurrent control must be reported at the same time. Abnormal fragility is indicated by hemolysis beginning at a concentration greater than 0.44 per cent, and by complete hemolysis at greater than 0.34 per cent. Poikilocytes show increased resistance to hemolysis, and spherocytes decreased resistance.

V. Indices and Mean Values.—Mean values and indices refer to the mean red cell or total erythrocyte characteristics and are of great clinical

PLATE I

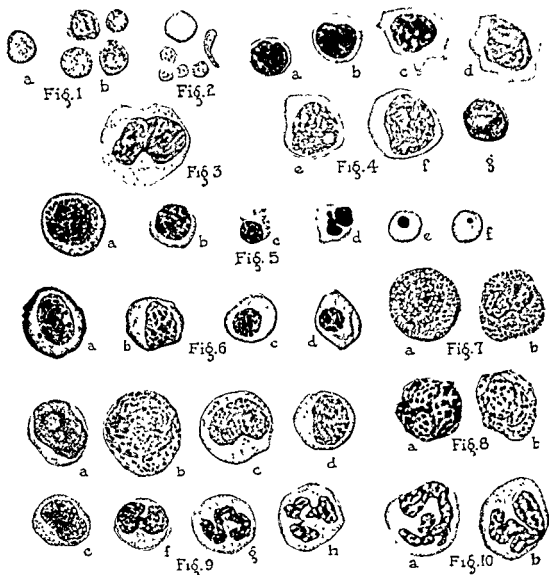


FIG. 1 — Reticulocytes from case of hereditary hemolytic jaundice. Supravital staining with brilliant cresyl blue. (a) Counter-stained with Wright's stain. (b) Without counter-stain.

FIG. 2 — Erythrocyte and blood platelets from normal blood.

FIG. 3 — Monocyte, normal blood.

FIG. 4 — Lymphocytes, a-d from normal blood; e-g from acute lymphatic leukemia. (a) Microlymphocyte. (b, c) Mesolymphocytes. (d) Macrolymphocyte. (e, f, g) Immature lymphocytes.

FIG. 5 — Normoblasts. (d) Karyorrhexis. (f) Jolly body.

FIG. 6 — Megaloblasts from pernicious anemia.

FIG. 7 — Eosinophil leukocytes. (a) Myelocyte with some dark granules. (b) Mature cell from normal blood.

FIG. 8 — Basophil leukocytes (mast leukocytes). (a) Myelocyte. (b) Mature cell.

FIG. 9 — Development.

From myelogenous leukocytes. (a) Myeloblast. (b) Myelocyte. (c) Myelocyte.

FIG. 10 — Atypical cells from subacute myelogenous leukemia. (a) Rieder cell. (b) Young neutrophil developed from a cell similar to (a).

value. They should not be computed unless they are derived from accurate data, all determined at the same time, or errors may be compounded. Allowance should be made for physiological variations, especially those associated with altitude. The indices are based on arbitrary normals of red blood count 5,000,000, hematocrit 43.2, and hemoglobin 14.5 gm.

1. Volume.—

$$\text{Volume Index} = \frac{\text{Hematocrit} \times 23}{\text{RBC in millions per c mm} \times 20}$$

$$\text{Mean Corpuscular Volume} = \frac{\text{Hematocrit} \times 10}{\text{RBC in millions per c mm}} = \text{cubic microns}$$

2. Corpuscle Thickness.—

$$\text{Mean Corpuscular Thickness} = \frac{\text{Mean corpuscular volume}}{3.14 \times \text{radius}^2} = \text{microns}$$

3. Hemoglobin.—

$$\text{Color Index} = \frac{\text{Hb (gm /100 cc} \times 6.9)}{\text{RBC in millions per c mm} \times 20}$$

$$\text{Mean Corpuscular Hemoglobin} = \frac{\text{Hb (gm /100 cc)} \times 10}{\text{RBC in millions per c mm}} = \text{micromicrograms (gamma gamma)}$$

$$\text{Saturation Index} = \frac{\text{Hb (gm /100 cc} \times 6.9)}{\text{Hematocrit} \times 23}$$

$$\text{Mean Corpuscular Hemoglobin Concentration} = \frac{\text{Hb (gm /100 cc)} \times 100}{\text{Hematocrit}} = \text{per cent}$$

The Mean Corpuscular Hemoglobin Concentration is not known to exceed a saturation of 36 per cent. Hence, higher computed values show error in hemoglobin or hematocrit determination. This is a convenient check and even a source of correction of methods by calculation.

1. Normal Values for Red Blood Cells, in Adults (after Wintrobe).—

	Red count (millions)	Hemoglobin (gm /100 cc)	Hematocrit
Females	4.8 ± 0.6	14.0 ± 2.0	42.0 ± 5.0
Males	5.4 ± 0.9	16.0 ± 2.0	47.0 ± 7.0

31 ± 2

VI. White Blood Cell Count.—The materials are the same as for the erythrocyte count except for the diluting fluid and the pipet. The fluid is a 0.5 per cent solution of glacial acetic acid in distilled water. This may be tinted with 1 drop of gentian violet for identification. Solutions should be freshly prepared every two weeks. The pipet has a smaller bulb than the red pipet, and usually contains a small white bead. The fifth line on the capillary tube is marked 0.5, the tenth 1.0 and above the bulb 11. In the procedure, blood is drawn from a large drop to the 0.5 mark, the tip cleared, and diluting fluid immediately added to the mark 11, with slight rotation of a vertical pipet. Then shake, discard 3 or 4 drops, and fill the counting chamber. Allow the cells to settle and examine under the low

power of the microscope. The white cells are counted in the four large corner squares (each 1 sq. mm. in area) in Figure 9. The maximum variation in the count of these squares should not exceed 25 per cent. Multiply the total number of cells in the four large squares by 50. This is the count per c.mm. Normal count is between 5000 and 10,000.

A lower count than 5000 is called leukopenia. A count higher than 10,000 (leukocytosis) is sometimes physiological. Counts in infants may range from 8000 to 20,000 in the absence of disease, especially in the first days of life. Strenuous exercise may give counts up to 30,000 with increase mainly

There
measle, ~~many, primary~~
comparative work, values should be determined under similar or basal conditions. The corrections of errors mentioned in the red count are applicable to this procedure.

VII. Platelets.—Blood platelets, or thrombocytes, are colorless, rounded, refractile bodies usually 2 to 4 microns in diameter. With ordinary staining methods, azure granules in hyaline light blue cytoplasm are seen. In active marrow regeneration, much larger forms are seen, and in films they readily deteriorate to form odd shapes that may be confused with parasites. Because of their fragility and tendency to agglutinate and to adhere to surfaces, an accurate count is most difficult.

1. Indirect Count.—This is made from the stained blood film by comparing the number of platelets with the already determined number of erythrocytes. The normal count is 200,000 to 300,000 per c.mm. A bleeding tendency may be assumed with counts under 50,000. This method is only roughly accurate.

2. Direct Count.—Use the hemacytometer previously described. Work rapidly.

Diluting fluid (Wright & Kinnicutt): Platelets appear lilac, the red cells are decolorized and the white cells are stained.

Aqueous solution brilliant cresyl blue (1:300)—2 parts.

Aqueous solution potassium cyanide (1:1400)—3 parts.

Keep in separate bottles. Mix and filter just before using. Cresyl blue solution is permanent, but may become contaminated with mold growth. The cyanide solution deteriorates in about ten days.

Procedure.—Draw diluting fluid to mark 1 on the erythrocyte pipet. Then draw blood from a fresh, freely bleeding puncture to the 0.5 mark and then diluting fluid to 101. The dilution is 1:200. Immediately mix by shaking for two minutes. Fill the chamber at once. Allow settling for ten minutes, then count. Always run a normal control with the same fluid. The values are lower than by the indirect count.

THE BLOOD FILM

In expert hands this is the most valuable part of the blood examination. Films should be prepared as described earlier. Its routine application is the ordinary differential count for leukocytes. Further value depends on

the training and experience of the observer. Descriptions of the cells are here held to a minimum and the reader is referred to the colored plate of the blood cells.

I. Routine Differential Count.—Using the oil immersion lens on a properly made, well-stained film, the field is scanned from one border of the slide to the other and all intact cells are tabulated. The report states the total number of cells counted and the percentages of each (eosinophils, basophils, neutrophils, lymphocytes, monocytes, and others). One may report the absolute number of each type of cell per c.mm. computed from the white blood count. The high dry power is not satisfactory for the count. A minimum of 200 cells should be counted.

Normal adult values are as follows:

Myelocytes	0
Neutrophilic metamyelocyte . . .	3-5
Neutrophil	51-62
Eosinophil	1-4
Basophil	0-1
Lymphocyte	25-33
Monocyte	3-7

From the neonatal period to age four or five years there are usually more lymphocytes than neutrophils. In general a lymphocytosis under 40 per cent is considered within normal limits.

II. Blood Cell Development.—There are many theories of blood cell development, well reflected in the confusion of nomenclature. In general it is accepted that some cells (myeloid) are derived from the bone marrow, others from the lymphatic tissues and perhaps others from the supporting tissues of the body.

The myeloblast is considered the stem cell of the marrow. Following the colored plate, the granulocytes (eosinophil, basophil or mast leukocyte, and neutrophil) develop in the order leukoblast, promyelocyte, myelocyte, metamyelocyte, and adult form, by a process of gradual transformation. The band form of the neutrophil is the stage occurring just prior to segmentation of the nucleus. Normal granulocyte regeneration is mainly at the myelocyte level. A giant multinucleated cell, also derived originally from the myeloblast, fragments its cytoplasm to form the blood platelets, according to the usual view. The erythrocyte normally regenerates at the normoblast level and passes through a series of cytoplasmic color changes (basophilic, polychromatophilic, normochromic) accompanied by pyknosis and fragmentation of its nucleus. Lymphocytes arise from other tissue lymphocytes, mainly in the lymph nodes and the spleen but also in the bone marrow itself. Monocyte origin is unsettled. Many feel that these cells may have myeloid, lymphoid, or reticulum cell origin.

Ordinarily with rapid loss or great demand, these cells appear in the peripheral blood in more immature forms. This is a physiological response.

III. Blood Film Study.—The blood film should always be examined systematically. A low-power view checks the adequacy of the preparation and also gives an estimate of the number of white blood cells. A high dry glance may be used for a rough differential, counting mononuclears and polymorphonuclears. The oil immersion lens should be used for cytologic study. It is a good rule to be systematic and examine all elements, red and

white cells, and platelets, in every case, and not to focus attention on one blood element to the exclusion of the others. Ordinarily the report should be descriptive and one is wise to avoid didacticism. If the case warrants further study, the quantitative data derived from the other procedures in the chapter may be determined. The following paragraphs briefly discuss cell morphology and related disease.

1. **Erythrocyte Morphology.**—The observations are ordinarily made on the stained film but for reliability may be repeated on the wet film. The normal cell is a biconcave disc, 1.9 to 2.1 μ thick. The average diameter is 7.5 μ . Measurements are most accurately made in a wet film preparation with an eyepiece micrometer, although for practical purposes the stained film is satisfactory. A Price-Jones curve is a graph to show the number of erythrocytes of different diameters. The occurrence of multiple peaks is indication of more than one red cell generation, as in pernicious anemia. Small cells are called microcytes, large cells macrocytes, while those of normal size are called normocytes. Variation in size is called anisocytosis. Increase in staining property (hyperchromasia) and decrease (hypochromasia) are related to the size and shape of the cell as well as to the hemoglobin content. Nucleated red cells, as well as bluish basophilia and bluish red polychromatophilia, are signs of immaturity. Basophilic stippling (common in lead poisoning) and nuclear fragments are commonly seen in pathological regeneration.

There may be marked variation in shape. If bizarre irregular forms occur it is called poikilocytosis. In hemolytic anemias, particularly familial hemolytic jaundice, many of the cells may be rounded (spherocytosis). In ovalocytosis, elliptical cells are seen. This is a familial trait and not a sign of disease. Seven to 8 per cent of negroes have the sickling trait in which the sealed wet film on standing for several hours develops crescent shaped red cells (sickle cells). Similar cells are found in the ordinary blood films of negroes with sickle cell anemia. The sickling trait alone is not diagnostic.

A morphological description of anemia is frequently of value. The erythrocyte picture is described as normocytic, macrocytic or microcytic, qualifying each term with normochromic, hyperchromic or hypochromic. *Normochromic anemias* are seen after acute hemorrhage and in hemolytic and aplastic anemias. Macrocytic anemias are usually of the pernicious anemia type, although they may result from intense bone marrow stimulation. Microcytic anemias, when of the normochromic form, are common in chronic diseases, and when of the hypochromic form, are commonly the result of iron deficiency.

The so-called pernicious anemia picture shows a macrocytic, apparently hyperchromic anemia in the more severe cases with megaloblasts (a nucleated red cell whose chromatin pattern is more open and delicate than that of the normoblast, and whose nuclear involution appears to be delayed later than in corresponding stages of the normoblast) and large hypersegmented neutrophils with cytoplasmic granulation more reddish than normal.

Polycythemia refers to an increase of the number of the red cells per c mm. This may be a normal response but in the chronic disease of polycythemia vera it is abnormal, amounting usually to 7 to 10 million, with resultant symptoms.

PLATE II

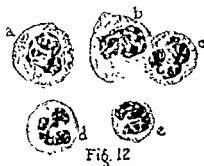


Fig. 15

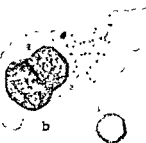


Fig. 16



Fig. 17

Parasites



Fig. 18

Fig. 11. Normal red cells. (a) Polkilocytes. (b) Macrocyte. (c) Polychromatophilus
 Fig. 12. cells (with
 Fig. 13. simplified
 Fig. 14. Fig.
 Fig. 15. Fig.
 Fig. 16. Fig.
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 (b) To
 Fig. 18. immo
 Fig. 18. — Trophozoite from the blood of a mouse
 (From Bell's Textbook of Pathology)

aped
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 cent.

2. Leukocyte Morphology.—Nucleated cells should be closely studied. Note cytoplasm for color, vacuoles, delicate azure granules, or specific granules. The nucleus is probably the more important part of the cell to observe. Always study the chromatin pattern for classification. The more immature cells generally have a more delicate nuclear pattern. Nucleoli are signs of immaturity and cells showing them are frequently called "blast" cells.

The neutrophil is the characteristic cell of the human species. Toxic effects are seen in shrinking and condensation of the nucleus with darker staining cytoplasmic granules.

The Schilling classification of polymorphonuclear leukocytes is a modification of the Arneth count in which the cells are graded according to the number of lobes of the nucleus. The neutrophils in the Schilling count are divided into four classes: the myelocytes, juveniles, bands, and segmenters.

An increase in band forms is the basis of the Schilling count and represents a regenerative "shift to the left." Similarly a hypersegmentation in the neutrophils with few younger forms is considered a sign of excessive maturity and is called a "shift to the right." In occasional cases the immaturity extends to myelocytes. A "leukemoid" picture is one in which the shift is far to the left with promyelocytes and even younger forms. This usually occurs with a leukocytosis and may resemble a myeloid leukemia, but it is not leukemic. A marked suppression of granulocytes is termed agranulocytosis.

The eosinophil is characterized by uniform large red granules. This cell is quite fragile and is frequently fragmented in a slowly dried film.

The basophil granules are water soluble, and when dissolved may leave what appear to be vacuoles in the cytoplasm.

Lymphocytes are of various appearances and sizes which need not be reported. Leukemia should be considered in the differential diagnosis if all cells seen are lymphocytes. In the large forms nucleoli may be vaguely outlined. As a whole the nuclear pattern appears relatively homogeneous

of the nucleus, with a more delicate nuclear pattern. The plasma cell has a deep blue cytoplasm with eccentric round nucleus containing wedges of coarse chromatin.

Leukemia is generally characterized by a high white count, and immature and atypical cells, in which cytoplasm and nucleus do not mature at the same rate. The Rieder cell has an immature nucleus, which is undergoing segmentation. Myelogenous leukemia is accompanied by a hyperplastic bone marrow and, in the chronic form, a peripheral white count of roughly 100,000 to 400,000. The leukocytes are mainly at the myelocyte level with myeloblasts increasing late in the disease. Nucleated red cells and increased numbers of basophils usually are present. There is a late secondary anemia. Acute myelogenous leukemia ordinarily shows a white cell count of 10,000 to 100,000. The cells are mainly near the myeloblast level. These cells are not always peroxidase positive. There is usually an associated acute anemia.

Lymphatic leukemia is generally accompanied by a hyperplasia of the

lymphoid tissues. In the chronic form the count averages 100,000, of which over 90 per cent are lymphocytes, generally showing nuclear immaturity. The anemia usually appears late in the course of the disease.

Monocytic leukemia is relatively uncommon. It appears to be either lymphoid or myeloid in origin. The immature cells in a case of leukemia are sometimes so primitive they cannot be classified. These are generally termed "stem cell leukemias." So-called aleukemic leukemia is almost always of the lymphatic type. In this variety of leukemia the total count is almost normal but careful study of the lymphocytes usually shows immaturity.

3. **Platelet Morphology.**—Platelets are difficult to study because of their small size. From experience one should know whether they are increased in number, about normal or decreased. Abnormal forms, such as giant masses are pathological. Platelets normally increase after hemorrhage and operations.

Thrombocytopenic purpura in the active stages is characterized by a low platelet count, usually under 50,000 per c.mm. The bleeding time is

tion time over five times normal, although the clot appears normal. The platelets appear normal in structure and number. The prothrombin time is usually much prolonged.

BONE MARROW

Bone marrow may be obtained by a biopsy, with excision of a piece of bone, which is then decalcified and treated as a surgical specimen, or more simply by aspiration of the sternum. The former method requires relatively radical surgical procedure with discomfort to the patient; when done imprints or streaks of the fresh marrow are made on slides for detailed study. In the aspiration method, after local anesthetic infiltration of skin and periosteum, either a special or improvised needle (18-gauge spinal puncture needle, with trocar) is passed into the sternum, and by suction on a syringe, 1 or 2 cc. of marrow aspirated. This method is simple, not particularly painful, and not dangerous. It has the disadvantage that the marrow may be diluted with sinusoidal blood.

After aspiration, the marrow in the syringe is mixed with heparin or a proper anticoagulant in a paraffin coated container. Films are made from the drop remaining in the syringe, from the marrow, which is best diluted with an equal volume of supernatant plasma, and from the buffy coat of the centrifugized specimen.

The centrifugation is done in the same manner as the hematocrit determination and the results are reported in detail. The report should include volume of marrow, packed cell volume, both as total and as percentage, the bottom red cell layer, the middle gray buffy coat of nucleated red cells and leukocytes, and the amount of fat. A differential count of 500 or 1000 cells is made from the film.

The principal value of an examination of the bone marrow, apart from indicating hypoplasia or hyperplasia, is found in a study of the film, showing as it does, any variations in the development of the different types of cells. The findings generally supplement the blood findings and must be

interpreted with reserve. The following table gives the approximate average normals of distribution, with normal maxima in parentheses.

TABLE 9.—NORMAL DISTRIBUTION OF CELLS IN BONE MARROW

Cell	Per cent
Myeloblast	1
Leukoblast	2
Promyelocyte	4 (8)
Myelocyte	10 (20)
Metamyelocyte (neutrophilic)	25 (35)
Neutrophil	20 (30)
Eosinophil (all types)	3 (8)
Basophil (all types)	1
Lymphocytes	10 (20)
Monocytes	1 (5)
Pronormoblasts	1
Normoblasts	22 (35)

In addition other cells such as plasma cells, reticulum cells, and a few megakaryocytes, will be found. The normal volume of nucleated cells is 6 to 8 per cent. Normal myeloid-erythroid ratio ranges from 1.75:1 to 3.75:1.

CHAPTER XI

HUMAN BLOOD GROUPS AND TRANSFUSION

By A. JAMES FRENCH

ARMY Regulations provide for the determination of blood groups of all enlisted and officer personnel as soon as possible after they have entered military service. The blood group is stamped upon the individual's identification tag so that in an emergency no time will be lost in regrouping, should the individual need a transfusion or if he is needed as a donor. Cross-matching prior to actual transfusion is indicated and done except in an extreme emergency.

The mass blood grouping of the present war-time Army has required larger quantities of grouping sera than are ordinarily needed and therefore dried rabbit sera of high titer have been developed and utilized in the present military program.

Satisfactory grouping sera will be of adequate titer to result in prompt agglutination of added corpuscles within thirty to sixty seconds. Proper attention to high titer of grouping sera will result in detecting subgroups A_1 , A_2 , etc., and thereby avoid reactions as indicated by Davidsohn.¹

HUMAN BLOOD GROUPS

I. Classification.—The International classification of blood groups has been accepted by the National Research Council and by the Army as the standard method. This obviates errors which may result from transposition of figures in the Moss and Jansky systems. The letters used indicate the presence or absence in the blood cells of isohemagglutinogens A and B. Inasmuch as neither A nor B agglutinin is present in group "O," this type is properly referred to as "zero." Usage, however, has resulted in reference usually being made to the letter "O." The following table compares the above systems:

TABLE 10—COMPARATIVE TERMINOLOGY OF BLOOD GROUPS

International	Moss	Jansky	Occurrence, per cent
O	IV	I	43
A	II	II	40
B	III	III	7
AB	I	IV	10

II. Grouping Sera.—1. **Preparation of Sera.**—Dried rabbit sera are prepared commercially by the immunization of rabbits against known group A and B human sera. The anti-A and anti-B rabbit sera thus produced are of higher titers than those found in ordinary pooled human sera and are stable when dried and kept in sealed containers.

Human grouping sera are obtained from known groups A and B individuals. Young healthy donors should be selected, as blood from such individuals normally has a greater agglutinating potency than that of young children or donors beyond middle age. The groups A and B bloods are collected separately to avoid error. The blood is collected under aseptic conditions, allowed to clot and the serum transferred to sterile containers. It is preferable to store the serum in small amounts in sealed

ampoules at 7° C., to avoid contamination of large amounts of serum in single containers. The use of preservatives has been advocated but they are not essential. These include phenol, acriflavine, brilliant green and merthiolate. The added advantage of coloring types A and B sera by the use of different colored preservatives may be desirable.

To lyophilize serum requires expensive bulky equipment and this precludes local preparation in most Army laboratories.

2. **Titration of Sera.**—In order to detect subgroups and to insure the accurate grouping of individuals with other groups of low agglutinin content, the grouping sera must contain sufficient agglutinin to agglutinate rapidly the red blood cells of the individual being grouped. The titer of a serum is frequently expressed as the reciprocal of the highest dilution of the serum at which agglutination occurs. For example, if agglutination is not present above a dilution of 1/40, after standing at room temperature for two hours, the titer is expressed as 40. Sera of potency less than a titer of 40 are usually not satisfactory.

III. **Determination of Human Blood Groups.**—The slide method is the most convenient and the one usually employed in the determination of blood groups. A plane glass slide is divided in halves by a wax pencil. Hollow ground slides may be used when small numbers of groupings are to be performed. In either event a ring of vaseline about the area which holds the blood cells prevents rapid drying of the preparation. The left, labeled "A" and the right, labeled "B," may be reversed in handling.

A suspension of red blood cells is prepared by adding 1 drop of blood to a test tube containing 2 cc. of physiologic salt solution. The subsequent technic will vary depending upon the type of grouping serum used.

1. Procedure with Rabbit Anti-serum.—If rabbit anti-serum is used, a drop of the cell suspension of the individual to be grouped is placed upon either side of the divided glass slide. A small amount of powdered anti-A rabbit serum on the end of a toothpick is added to the cell suspension on the left or "A" side of the slide and mixed. A similar amount of powdered anti-B rabbit serum is added to the cell suspension on the right or "B" side of the slide and mixed. The reactions which occur using such rabbit grouping sera are:

serum and anti-B rabbit serum

2. **Procedures with Human Grouping Sera.**—If human grouping sera are used, a drop of A serum is placed on the left side of the clean glass slide and a drop of B serum on the right side. A drop of the red blood cell suspension of the individual being grouped is mixed with the group A human serum of the blood with . Care must be taken to observe the agglutination mixtures. The reactions which occur using human grouping sera are:

A or B serum

The titer of either the rabbit or human serum must be high enough to cause agglutination of the red cells within one minute. Agglutination should be complete in five minutes' time, but thirty minutes should elapse before a final recording is made. Agglutination can be differentiated from rouleaux formation by agitation of the slide to break up rouleaux. Agitation hastens agglutination.

IV. Other Agglutinogens.—In addition to the four main groups of agglutinogens O, A, B, and AB, in human blood, there exist agglutinogens A₁, A₂, and M and N. The finding that absorption of group B serum with the group A cells of certain individuals failed to remove agglutinins for the cells of certain other group A individuals lead to the description of the A₁ and A₂ sub-groups of group A. These subgroups, found also in group AB persons, are sometimes of value in medico-legal cases, and are also the basis of some transfusion reactions.

M and N agglutinogens were established when Landsteiner and Levine³ found that certain sera from rabbits immunized against human blood contained agglutinins in addition to anti-A and anti-B agglutinins. Three distinct types of human blood occur depending upon the occurrence of M or N or both M and N agglutinogens. The agglutinogens M and N are unrelated to A and B agglutinogens as the distribution of M, N or MN types of human blood is the same in each of the four blood groups. A later reference will indicate the application of the M and N agglutinogens to medico-legal cases.

V. Cross-matching Donors for Transfusion.—The selection of donors for transfusion requires matching the corpuscles and serum of the donor and recipient against each other. A drop of blood from the finger or ear of the recipient is placed in a small test tube containing 2 cc. of physiologic salt solution, and enough blood taken by venepuncture to yield some serum. Similar preparations are made from each prospective donor, all being appropriately labeled. Cross-matchings are set up separately for the recipient's cells mixed with each donor's serum and also for each donor's cells with the recipient's serum. Thus, if there are three prospective donors, 6 mixtures of serum and cells are prepared.

More in detail, the technic is as follows: A glass slide is divided into right and left halves with a wax pencil. The left half is marked "DS" (donor's serum) and the right is marked "RS" (recipient's serum). A drop of the donor's serum is placed in a vaseline ring on the left side of the slide and a drop of the recipient's serum is placed on the right side of the slide. A drop of recipient's cells is placed in the donor's serum and a drop of donor's cells is added to the recipient's serum. The slides are kept at room temperature and examined every few minutes for agglutination. Periodic agitation of the slides tends to break up rouleaux formation and accelerate agglutination. Clumping usually occurs within a few minutes but thirty minutes should elapse before the final reading is recorded. Macroscopic and microscopic reading of slides for agglutination are recommended, depending upon the degree of agglutination, and the experience of the technician. Any prospective donor whose cells are agglutinated by the recipient's serum or whose serum agglutinates the recipient's cells is ordinarily rejected if a more suitable donor is available. Agglutination of donor's cells by recipient's serum is of more importance than the reverse situation, so that, in case of necessity, Group O donors

may be used for recipients of other groups. Provided that the rate of injection of the transfused blood is very slow, the agglutinin present in the donor's serum ordinarily will be diluted enough by the recipient's serum to preclude agglutination of the recipient's cells by the donor's serum.

The test tube method of blood grouping and cross-matching has been advocated as superior to the slide method but the claimed advantages are not considered sufficient to warrant its adoption as the routine Army method for mass grouping and cross-matching.

VI. Medico-Legal Application of Blood Groups.—Inasmuch as blood cell agglutinogens are inherited in accordance with the Mendelian Law, considerable medico-legal significance has been attributed to blood groups.

Only negative information is obtainable, however, as a child's cells will not contain agglutinogens not present in the blood cells of one or the other of the parents. Inasmuch as A and B agglutinogens are inherited as dominant characteristics, no child will exhibit agglutinogens not present in the cells of one of the parents, but it does not follow that all of the agglutinogens present in the cells of the parents will appear in the blood cells of the offspring. The M and N agglutinogens behave similarly, and are therefore of similar significance, as described by Wiener.⁶

Blood groups are also of value in the identification of blood stains in medico-legal and other cases. In paternity cases, only the absence of agglutinogens in the blood of the accused can be submitted as evidence.

VII. The Rh Factor in Human Blood.—The search for additional agglutinogens in human blood led to the discovery of the Rh factor by Landsteiner and Wiener⁴ as a result of immunizing rhesus monkeys with human blood. The designation Rh was selected to indicate rhesus monkeys as the source of the substance. The blood of about 85 per cent of all white individuals contains an agglutinable factor with the Rh substance produced in rhesus monkeys. This factor in human blood may be of significance when patients receive repeated transfusions. If an Rh negative recipient receives Rh positive blood, he may become sensitized to the Rh factor by repeated transfusions and may exhibit a severe hemolytic reaction, when transfused again. Rh negative donors must be selected to transfuse such persons safely. The blood of mothers of some infants with erythroblastosis fetalis contains the Rh agglutinin.

The technic and interpretation of the method of typing for the Rh factor has been described by Landsteiner and Wiener.⁵ The Rh factor should be considered when hemolytic reactions, otherwise unexplainable, occur in individuals receiving repeated transfusions. When due to Rh agglutination, the first such reaction is usually mild but the severity increases following subsequent transfusions. It is not believed that such reactions will constitute a serious military problem.

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For details concerning the preparation and standardization of Kahn antigen see "The Kahn Test, A Practical Guide," by R. L. Kahn, Williams & Wilkins Company, Baltimore, 1928.

2. *Saline Solution*.—This solution consists of 0.9 per cent sodium chloride in distilled water. The sodium chloride must be chemically pure and the solution filtered before being used. The sodium chloride should be dry before weighing. It may be expedient to weigh out 4.5 gm. or 9 gm. of thoroughly dried sodium chloride into individual test tubes and then dissolve the contents in 500 cc. or 1000 cc. quantities of distilled water, respectively, just before use.

3. *Serum*.—Separate serum from the clot by centrifugation. Care must be exercised to insure complete removal of blood cells. These particles might give the impression of a precipitate in the completed test. The test is not affected by sera showing some hemolysis or bile, but those showing marked hemolysis, bacterial contamination or chyle are not suitable for testing. Specific directions for the collection and preparation of specimens for serologic examination will be discussed elsewhere in this book.

The serum, after it has been heated for half an hour at 56° C., is examined for the presence of particles. If present, the serum is cleared by recentrifugation.

4. *Spinal Fluids*.—Spinal fluid is centrifugalized to render it free from cells and foreign particles.

III. *Standard Test with Serum*.—1. *Preparation of Standard Antigen Suspension*.—This suspension is prepared when the sera are taken from the 56° C. water bath. Antigen is mixed with salt solution according to the required titer. Thus, if the titer is 1 cc. antigen plus 1.2 cc. physiologic salt solution, the antigen is mixed as follows: (1) 1.2 cc. salt solution are measured into a chemically clean and dry standard antigen suspension vial; (2) 1 cc. antigen is measured with a chemically clean and dry pipet into a similar vial; (3) the salt solution is poured into the antigen, and as rapidly as possible, without waiting to drain the vial, the mixture is poured back and forth six times to insure thorough mixing; (4) the antigen suspension is allowed to stand for ten minutes before using. The suspension must be used within thirty minutes from the time of mixing. An old antigen suspension is not to be mixed with a newly prepared suspension.

More than 1 cc. of antigen may be mixed with a proportionately larger amount of salt solution. Thus, in case of an antigen with the above titer, 2 cc. may be mixed with 2.4 cc. salt solution and 2.5 cc. with 3 cc. salt solution. Do not use amounts of antigen less than 1 cc. or more than 2.5 cc. for the preparation of an antigen suspension.

2. *Measuring Antigen Suspension*.—After standing ten minutes the antigen suspension is shaken well, and distributed into 3 tubes for each serum to be tested, in amounts of 0.05, 0.025 and 0.0125 cc. delivered to the bottom of the tubes. The standard rack capacity is 30 tubes; the 0.05 cc. amounts are measured into the tubes of the first row, the 0.025 cc. amounts in the tubes of the second row, and the 0.0125 cc. amounts in the tubes of the third row.

3. *Measuring Serum*.—The serum is added as soon as possible after the antigen suspension has been pipetted to avoid undue evaporation of the suspension. When examining large numbers of sera, it is well for one worker to measure the antigen suspension and for another to follow with the

serum. Each serum, in 0.15 cc. amounts, is added to the 0.05, 0.025, and 0.0125 cc. amounts of antigen suspension, and the rack of tubes is shaken vigorously for ten seconds to insure thorough mixing of the ingredients. The serum-antigen mixtures should stand for about five to seven minutes (preferably not less than three minutes and not more than ten minutes) at room temperature before the mechanical shaking for three minutes.

4. **Controls.**—Set up three separate tests, one with a positive serum, one with a negative serum and one employing salt solution instead of serum. The antigen suspension for these controls should be pipeted immediately before pipeting the antigen suspension for the tests; the serum of these controls should be added immediately before the serums under test.

5. **Shaking.**—The tests and controls are shaken in a standard shaking machine for three minutes. The machine oscillates 275 to 285 times per minute, with a stroke of 1½ inches.

6. **Addition of Salt Solution.**—After the three-minute shaking period, 1 cc. of salt solution is added to each tube of the first row of the rack (containing the 0.05 cc. amounts of antigen suspension) and 0.5 cc. salt solution to the remaining tubes. The rack is shaken by hand sufficiently to mix the contents.

7. **Reading.**—If Kahn flocculation test results (as ...), proper training, experience and lighting arrangements are necessary. The use of magnification by means of the microscope mirror may be of assistance. Beginners should read as many tests as are available in comparison with an experienced reader, until they can read correctly and with ease, before they assume the responsibility of reading and reporting results to physicians.

Uniformity in the reading of results will be greatly aided if all Kahn tests are read with the aid of the concave surface of a microscope mirror and a constant source of light from a microscope lamp. A two- to three-fold magnification obtained by holding the tube 2 to 3 inches above the mirror will give sufficient magnification, yet be low enough to avoid the visualization of non-specific particles in the negative tests.

8. **Interpretation of Results.**—The following scheme for interpretation of Kahn results has been devised School, Army Medical Center, based on two readings of the K iately after the addition of salt minutes later, the racks remaining at room temperature during the interval. The final interpretation is based on the total number of pluses resulting from both readings, the plus-minus (\pm) reactions being disregarded, as follows:

0 to 3 pluses, inclusive	= Negative, e. g., ± 2 ; ± 1
4 to 12 pluses, inclusive	= Doubtful
13 to 24 pluses, inclusive	= Positive

9. **Final Report.**—The final report to the clinician should be reported as negative, doubtful or positive, according to the above scheme, but the actual tube readings should be recorded in the laboratory.

10. **Supplementary Tests.**—An atypical type of precipitation reaction is met with occasionally, in which precipitation is marked in the first tube (front row), and weak or negative in the second (middle row) or third

(back row) tube. In this instance, the serum generally is so rich in antibody that it requires a relative excess of antigen suspension to give maximum precipitation. When a reaction of this type is encountered, it is necessary to set up a supplementary test in which the amount of antigen suspension in relation to serum is increased beyond that employed in the standard test. A supplementary test is set up in which 2:1 and 1:1 proportions of serum to antigen suspension are used, thus:

	Tube 1	Tube 2
Antigen suspension, cc.	0.025	0.025
Serum, cc.	0.025	0.05
Shake tests 3 minutes		
Salt solution, cc.	0.3	0.3

These two tubes, or at least Tube 2, should show definite flocculation, if the serum is strongly positive.

As an additional check on serums giving precipitation reactions in the first tube of the standard test and negative reactions in the remaining two tubes, a second supplementary test is made by setting up a partial quantitative test. Thus, dilute the serum 1:5, 1:10, and 1:20 with salt solution and examine each dilution with antigen suspension in a proportion of 15:1, in accordance with the following outline:

	Tube 1	Tube 2	Tube 3
Antigen suspension, cc.	0.01	0.01	0.01
Diluted serum, cc.	0.15 (1:5)	0.15 (1:10)	0.15 (1:20)
Shake tests 3 minutes			
Salt solution, cc.	0.5	0.5	0.5

If one or more tubes show definite flocculation, the serum should be considered positive.

If these supplementary examinations do not show positive reactions, then the three-tube test which shows marked precipitation in the first tube and negative precipitation in the remaining two tubes must be considered as giving a weak or doubtful reaction.

Rarely one finds that the three-tube tests show a borderline precipitate in each of the three tubes, such as =, =, =; +, +, —, or perhaps ++, ++, ++. After ascertaining that these borderline reactions are not due to serum particles, the two supplementary tests described above are carried out. If these two tests show definite flocculation, the tests are reported as positive. If the supplementary tests are also borderline or questionable, the reaction may be non-specific, due to conditions other than syphilis.

QUANTITATIVE KAHN TEST

Quantitative serological tests for syphilis yield information of value from a clinical standpoint only under special circumstances and should not be made routinely. A quantitative test is ordinarily significant only when considered in relationship to similar tests which have preceded it or which may be made at a later date.

I. Procedure.—1. *Dilution of Positive Serum with Salt Solution.*—A series of serum dilutions with physiologic salt solution is prepared so that the ratio of the volume of diluted serum to the volume of serum before dilution

ranges from 5 (1 part serum plus 4 parts salt solution) to 60 (1 part serum plus 59 parts salt solution). The following scheme is employed:

Dilution No.	Dilution ratio
(1)	1 = 0.2 cc. undiluted serum
(2)	5 = 0.2 cc. undiluted + 0.8 cc. salt solution
(3)	10 = 0.7 cc. of (2) + 0.7 cc. salt solution
(4)	20 = 0.2 cc. of (3) + 0.2 cc. salt solution
(5)	30 = 0.2 cc. of (3) + 0.4 cc. salt solution
(6)	40 = 0.1 cc. of (3) + 0.3 cc. salt solution
(7)	50 = 0.1 cc. of (3) + 0.4 cc. salt solution
(8)	60 = 0.1 cc. of (3) + 0.5 cc. salt solution

2. **Performance of Test.**—The serum dilutions being ready, the antigen suspension is prepared in the usual manner. After ten minutes standing, it is pipeted in 0.01-cc. amounts into each of 8 standard test tubes, depositing the suspension at the bottom of the tubes. With an appropriate pipet, 0.15-cc. amounts of the 7 serum dilutions are added in order to the tubes containing antigen suspension, beginning with the highest dilution (8). The racks are shaken for three minutes in the usual manner, 0.5 cc. saline is added to each tube and the results are read.

3. **Determination of Kahn Units.**—If a serum gives a + + + +, + + +, or + + reaction in an undiluted state only, it is said to contain the number of Kahn units indicated by the plus signs (4 units, 3 units, or 2 units, respectively). The titer of any serum which is positive on dilution is determined according to the formula $S = 4D$, where S is the serum titer in terms of Kahn units and D is the highest dilution ratio giving a positive (+ + + +) reaction. Thus, if serum dilution 1:5 is four plus and 1:10 and higher dilutions are less than four plus, the serum contains 5×4 or 20 Kahn units. If serum dilution 1:10 is four plus and 1:20 and higher dilutions are less than four plus, the serum contains 40 units.

4. **Reporting Results.**—Results are reported "4 Kahn units," "40 Kahn units," etc., as the case may be.

5. **Highly Potent Sera.**—If a serum gives a four plus reaction with a dilution ratio of 60, still higher dilutions of serum are examined with antigen suspension until four plus reactions are no longer obtained. Higher dilutions are readily prepared from Tube 3 in the dilution series (1:10 dilution), an excess of which is available.

KAHN TEST WITH SPINAL FLUID

In the testing of spinal fluid, flocculation tests are believed to be less reliable than complement-fixation tests, since false positive reactions tend to occur with the former. The Kahn flocculation test is recommended, therefore, only for smaller laboratories which are not equipped to do Wassermann tests and where there is danger of the spinal fluid being contaminated while in transit to a distant laboratory.

A negative Kahn test on the spinal fluid may be reported as such, whereas doubtful and positive results should be confirmed whenever possible by a complement-fixation test. When facilities are available for confirming the spinal fluid Kahn test by a complement-fixation test the standard Kahn test as outlined below may be employed. In unusual circumstances where no confirmation is possible a quantitative Kahn test should be made on the spinal fluid.

I. Standard Kahn Test with Spinal Fluid.—In this test, the spinal fluid globulins are precipitated by half saturation with ammonium sulfate, and redissolved in an amount of physiologic salt solution equivalent to one-tenth of the original spinal fluid volume. The concentrated globulin solution thus obtained is then tested with standard antigen suspension.

1. Preparation of Concentrated Globulin Solution.—(a) *Reagents.*—The reagents needed for the preparation of concentrated globulin solution are (1) spinal fluid, (2) a saturated solution of ammonium sulfate of the highest purity, and (3) physiologic salt solution.

(b) Spinal fluid is centrifugalized to free it of cells and foreign particles.

(c) Place 1.5 cc. of the clear fluid in a standard Kahn test tube (7.5 by 1 cm.).

(d) To the same tube add 1.5 cc. of a saturated solution of ammonium sulfate.

(e) The fluids are mixed by covering the mouth of the tube with the thumb (protected with rubber) and shaking back and forth vigorously. The mixture is placed in a 56° C. water bath for fifteen minutes to hasten the precipitation of the globulins.

(f) Centrifugalize the mixture at high speed (about 2000 r.p.m.) for fifteen minutes to throw down the precipitated globulins completely.

(g) The supernatant fluid is removed as completely as possible with a finely-drawn capillary pipet. An alternative method for removing the supernatant fluid is to pour off the fluid and invert the tube in a standard rack over clean filter paper, allowing the moisture that adheres to the tube to drain and be absorbed by the paper for a period of ten minutes. This draining period is not necessary if the inside of the tube is wiped dry by means of filter paper. The filter paper is first wound around a glass rod or pencil and is then inserted into the tube, without touching the precipitate. Fresh filter paper is employed for wiping each tube.

(h) Add 0.15 cc. of salt solution to the precipitate, and redissolve by gentle shaking. In adding this salt solution, the point of the pipet is held close to the bottom of the tube to avoid washing down traces of ammonium sulfate that may adhere to the inner wall.

(i) This globulin solution after being examined for clarity and freedom from particles is ready to be tested with antigen suspension.

2. Preparation of Antigen Suspension.—Mix salt solution with antigen in the same manner as for the standard test with serum. The antigen suspension is allowed to stand ten minutes and must be used in the test within the next twenty minutes.

3. Measuring Antigen Suspension.—With a 0.2-cc. pipet graduated to 0.001 cc., 0.01 cc. of antigen suspension is measured to the bottom of a standard Kahn test tube.

4. Measuring Concentrated Globulin Solution.—With another 0.2-cc. pipet, add 0.15 cc. of the concentrated globulin solution. Tubes are shaken vigorously for ten seconds to mix ingredients.

5. Controls.—Positive and negative spinal fluid controls are included.

6. Shaking.—After mixing, allow the tubes to stand at room temperature for three minutes before shaking at the standard speed for four minutes.

7. Addition of Salt Solution.—Add 0.5 cc. of physiologic salt solution to each tube.

8. **Reading Results.**—Four-plus, three-plus, and two-plus reactions are reported as positive; one-plus reactions are reported as doubtful and plus-minus and negative reactions are reported negative. If the spinal fluid contains blood it should be so noted on the report.

9. **Check Examinations.**—Each spinal fluid test should be performed in duplicate. Hence, the amount of spinal fluid required for a test is a little over 3 cc.

II. **Quantitative Kahn Test for Spinal Fluid.**—1. **Concentration of Globulin.**—Prepare concentrated globulin solution from spinal fluid as directed above.

2. **Antigen Suspension.**—Prepare as directed above.

3. **Dilutions of Concentrated Globulin.**—While the antigen is aging, make dilutions of the globulin solution as follows, using five tubes:

Dilution No.	Dilution ratio of concentrated globulin
(1)	This is the 0.3 cc. of globulin solution in the centrifuge tube
(2)	5 = 0.15 cc. of (1) + 0.6 cc. salt solution
(3)	10 = 0.4 cc. of (2) + 0.4 cc. salt solution
(4)	20 = 0.2 cc. of (3) + 0.2 cc. salt solution
(5)	40 = 0.2 cc. of (4) + 0.2 cc. salt solution

4. **Procedure for Test.**—This is a five-tube test. Place five Kahn tubes in the rack in a row.

(a) Pipet 0.01 cc. of antigen that has stood for ten minutes into each tube.

(b) Pipet 0.15 cc. of each globulin dilution into the tubes containing the antigen, beginning with the last tube. Mix and allow to stand for three minutes at room temperature.

(c) Shake three minutes at standard speed, then add 0.5 cc. of salt solution.

5. **Reporting Results.**—Results may be reported in Kahn Units by multiplying the greatest dilution giving a four-plus reaction by 4. A fluid giving a four-plus reaction in the 1:10 dilution may be reported as having 40 Kahn Units.

KOLMER-WASSERMANN COMPLEMENT-FIXATION TEST WITH SERUM AND SPINAL FLUID

I. **Glassware and Apparatus.**—1. **Pipets.**—The following pipets are recommended:

1 cc. graduated in 0.01 cc. to tip.

5 cc. graduated in 0.10 cc.

10 cc. graduated in 0.10 cc.

2. **Test Tubes.**—For the routine simplified test, which has a total volume of 2.7 or 3 cc., test tubes measuring 10 by 1.3 cm. (inside diameter) with rounded bottoms and no lips are employed.

3. **Cylinders.**—Graduated (100 and 250 cc. capacity) to be used for measuring amounts over 50 cc.

4. **Test-tube Racks.**—Galvanized wire racks carrying 12 rows of 6 tubes each.

5. **Water Bath.**—Any easily regulated water bath which can be used satisfactorily at 56° or 37° C. is suitable.

6. **Refrigerator.**—Any refrigerator maintaining a temperature of 6° to 5° C. is satisfactory.

II. Reagents.—1. **Kolmer Saline Solution.**—Dissolve 8.5 gm. of dry, chemically pure sodium chloride and 0.1 gm. magnesium sulfate in 1000 cc. of freshly distilled water. If the salt has absorbed moisture it should be dried in the hot-air oven for ten or fifteen minutes before weighing. Filter the solution through paper into a flask fitted with a gauze-covered cotton stopper

2 **Sheep Cells.**—Blood may be obtained by bleeding a sheep from the external jugular vein. In a clean and preferably sterile 1-liter container, place 30 cc. of a 10 per cent solution of sterile sodium citrate. Two cc. of formalin may be added as a bacteriostatic agent. Fill three-quarters full with fresh blood, stopper, mix well with the citrate solution, and keep in a refrigerator. Alternatively, the sheep blood may be collected in a dry flask containing a handful of sterile glass beads, and defibrinated by shaking. Keep blood for forty-eight hours in the refrigerator before using. Sheep blood usually remains satisfactory for use over a period of two weeks, but as soon as the corpuscles become too fragile a fresh supply should be secured. The cells may be kept for a longer period if an equal volume of 5 per cent dextrose in Kolmer saline is added to the freshly citrated or defibrinated blood.

Filter a small quantity of blood through a cotton-gauze filter into a graduated centrifuge tube. Add 2 or 3 volumes of saline solution. Centrifugalize at a moderate speed until all the corpuscles are thrown down.

Remove the supernatant fluid with a capillary pipet or by suction. Add 3 or 4 volumes of saline solution; mix by inverting and again centrifugalize for the same length of time. Repeat the process for a third time but centrifugalize at 1000 r.p.m. for exactly fifteen minutes for the final packing of cells. It is important that these two factors be kept constant in order that the cell suspensions may be uniform from day to day. Cells should be washed until the supernatant fluid is almost colorless. Three washings are usually sufficient. If more than four washings are necessary, the cells are too fragile for use.

Read the volume of cells in the centrifuge tube, carefully remove the supernatant fluid, and prepare a 2 per cent suspension by washing the cells into a flask with 49 volumes of saline solution. Always shake before using to secure an even suspension, as the corpuscles settle to the bottom of the flask on standing.

3 **Sera.**—Separate serum from the clot and centrifugalize until entirely free of cells. Inactivate in a water bath at 56° C. for thirty minutes.

4. **Spinal Fluids.**—Usually spinal fluids must be centrifugalized, but they need not be inactivated since they contain but little complement. Bloody fluids are unsatisfactory for testing since positive results may be due to the activity of the serum rather than the spinal fluid. Spinal fluids more than three days old, or contaminated with bacteria, may be heated at 56° C. for fifteen minutes to destroy thermolabile anticomplementary substances.

5. **Egg Albumin.**—This modification may be employed in the spinal fluid technic when prozone reactions are obtained. Break a fresh egg and separate the white from the yolk. Discard the yolk. Pick out heavy particles or filter through several layers of gauze. Measure the egg-white and beat briefly before adding to an equal volume of normal saline. In the tests this 50 per cent solution may be used in 0.2 cc. amounts. An

alternative method is to prepare a 10 per cent solution in Kolmer saline solution and to use this for diluting the complement (1 cc. to carry 2 full units). This 10 per cent solution of egg albumin may be prepared by diluting each 10 cc. of albumin with 90 cc. of saline solution, or by diluting 20 cc. of the 50 per cent solution with 80 cc. of saline solution. Egg-white solutions should be made fresh for each day's use and placed in the refrigerator until needed.

6. **Antigen.**—An alcoholic extract of ether-extracted powdered beef heart, containing 0.4 per cent cholesterol. The antigen is customarily prepared in a central laboratory and its anticomplementary, hemolytic and antigenic titer determined before shipment to field installations. Since the antigen usually remains stable over long periods of time frequent titrations of antigen are unnecessary. When a new lot of antigen is received it is desirable to check its hemolytic, anticomplementary and antigenic titer.

7. **Complement.**—Complement may be prepared in the individual laboratory by pooling the clear fresh sera of at least three guinea pigs. Select large healthy animals which have not been fed for twelve hours. Avoid pregnant animals. Bleed from the heart. Unless preserved by freezing or by desiccation the serum should not be used after twenty-four hours, since its potency even at icebox temperature decreases rapidly. Immediately before using dilute 1 cc. with 29 cc. of normal saline solution, giving a 1:30 dilution.

Fresh serum may be preserved for longer periods by adding 0.25 gm. of chemically pure sodium chloride per cc. serum. It is then stored in the freezing compartment of a mechanical refrigerator. To prepare for use, dilute 1 cc. with 29 cc. of distilled water, thus making a 1:30 dilution of serum in 0.85 per cent salt solution.

In small laboratories it is desirable to use complement which has been desiccated *in vacuo* and supplied from some central laboratory. Directions for rehydrating will accompany the ampules of dried complement.

8. **Hemolysin (Amboceptor).**—An antisheep hemolysin contained in the serum of rabbits which have been injected with sheep red blood cells. Amboceptor is preferably supplied from a central laboratory, but it may be produced locally. The following procedure for producing amboceptor gives satisfactory results:

Intravenous injections of washed packed sheep cells are made at the following intervals:

Day	Dose	Dilution
1st	0.1 cc. packed cells	1.0 cc. of 10% suspension
2d	0.2 cc. packed cells	2.0 cc. of 10% suspension
4th	0.4 cc. packed cells	4.0 cc. of 10% suspension
7th	0.6 cc. packed cells	6.0 cc. of 10% suspension
11th	0.8 cc. packed cells	8.0 cc. of 20% suspension
14th	1.0 cc. packed cells	1.0 cc. of 20% suspension
22d	Bled for preliminary titration	

If the preliminary titration is satisfactory (at least 1:4000) the rabbit is bled from the heart and the serum diluted with an equal volume of glycerol. It is of utmost importance to use neutral chemically pure glycerol, otherwise the hemolysin may undergo deterioration. If the preliminary titration is unsatisfactory, the rabbit should be given several additional injections of sheep cells. In preparing the stock solution, take into consideration the fact that the hemolysin is mixed with equal parts of glycerol.

III. Titrations.—1. Titration of Hemolysin (Amboceptor).—It is advisable to repeat the hemolysin titration for each series of complement-fixation tests.

(a) Prepare a stock dilution of 1:100 hemolysin as follows:

Phenol (5 per cent in saline solution)	4 cc.
Saline solution	94 cc.
Glycerolized hemolysin (50 per cent)	2 cc.

The phenol and saline solutions should be well mixed before glycerolized hemolysin is added. This stock solution should be kept in the refrigerator. For titration, prepare a 1:1000 solution (0.5 cc. of stock 1:100 dilution + 4.5 cc. saline).

(b) In a series of 10 tubes, prepare higher dilutions as follows:

No. 1	0.5 cc. hemolysin (1:1,000)	1: 1,000
No. 2	0.5 cc. hemolysin (1:1,000) + 0.5 cc. saline solution	1: 2,000
No. 3	0.5 cc. hemolysin (1:1,000) + 1.0 cc. saline solution	1: 3,000
No. 4	0.5 cc. hemolysin (1:1,000) + 1.5 cc. saline solution	1: 4,000
No. 5	0.5 cc. hemolysin (1:1,000) + 2.0 cc. saline solution	1: 5,000
No. 6	0.5 cc. hemolysin (1:1,000) + 2.5 cc. saline solution	1: 6,000
No. 7	0.5 cc. hemolysin (1:1,000) + 3.0 cc. saline solution	1: 8,000
No. 8	0.5 cc. hemolysin (1:1,000) + 3.5 cc. saline solution	1:10,000
No. 9	0.5 cc. hemolysin (1:1,000) + 4.0 cc. saline solution	1:12,000
No. 10	0.5 cc. hemolysin (1:1,000) + 4.5 cc. saline solution	1:16,000

Mix the contents of each tube thoroughly.

(c) Prepare a 1:30 dilution of complement for hemolysin and complement titration by diluting 0.2 cc. of fresh complement serum with 5.8 cc. of saline solution (or 0.2 cc. of salted serum with 5.8 cc. of distilled water).

(d) Prepare a 2 per cent suspension of sheep cells.

(e) In a series of 10 tubes set up the hemolysin titration as shown in the following table:

TABLE 11.—TITRATION OF HEMOLYSIN

Tube	Hemolysin, 0.5 cc.	Complement (1:30), cc.	Saline solution, cc.	2 per cent sheep cells, cc.
1	1: 1,000	0.3	1.7	0.5
2	1: 2,000	0.3	1.7	0.5
3	1: 3,000	0.3	1.7	0.5
4	1: 4,000	0.3	1.7	0.5
5	1: 5,000	0.3	1.7	0.5
6	1: 6,000	0.3	1.7	0.5
7	1: 8,000	0.3	1.7	0.5
8	1:10,000	0.3	1.7	0.5
9	1:12,000	0.3	1.7	0.5
10	1:16,000	0.3	1.7	0.5

Mix the contents of
for one hour. Read the
of hemolysin that gives
give a unit of 0.5 cc. of 1:4000 or higher dilution.

Two units of hemolysin are used in titra-
tions and in the complement-fixation that
0.5 cc. contains 2 units. For example 1:6000
dilution, two units equal 0.5 cc. of the 1:3000 dilution. Dilute just enough
hemolysin for the complement-titration and the complement-fixation tests.
Keep hemolysin and sheep cells in suspension in the refrigerator when not
in use.

The following table shows how to prepare hemolysin dilutions so that 0.5 cc. carries two units:

TABLE 12.—DILUTION OF HEMOLYSIN

1 unit 0.5 cc. of	2 units would be 0.5 cc. of	Prepared by diluting 1 cc. of stock 1:100 with following amounts of saline, cc.
1: 4,000	1:2,000	19
1: 5,000	1:2,500	24
1: 6,000	1:3,000	29
1: 8,000	1:4,000	39
1:10,000	1:5,000	49
1:12,000	1:6,000	59
1:16,000	1:8,000	79

In practice the hemolysin titration may be placed in the water bath at the same time as the complement titration. At the end of the first incubation of the complement titration the unit of hemolysin is available and two units are added to all the tubes of the complement titration, etc.

2. **Titration of Complement.**—For the complement titration use the 1:30 dilution of complement. Dilute antigen so that the dose employed in the main tests is contained in 0.5 cc. (see Section IV below). The required amount of saline solution is placed in a flask and antigen is added drop by drop, with shaking. Prepare enough antigen dilution for the complement-titration and the complement-fixation tests.

In a series of 8 test tubes, set up the complement titration as follows:

Tube	Complement (1:30), cc.	Antigen dose, cc.	Saline solution, cc.	Hemolysin (2 units), cc.	Sheep cells (2 percent), cc.
1	0.2	0.5	1.3	0.5	0.5
2	0.25	0.5	1.3	0.5	0.5
3	0.3	0.5	1.2	0.5	0.5
4	0.35	0.5	1.2	0.5	0.5
5	0.4	0.5	1.1	0.5	0.5
6	0.45	0.5	1.1	0.5	0.5
7	0.5	0.5	1.0	0.5	0.5
8	None	None	2.5	None	0.5

Water bath, 37° C.
for 1 hour

Water bath, 37° C.
for 1 hour

The smallest amount of complement just giving complete hemolysis is the exact unit. The next larger quantity is the full unit, which contains 0.05 cc. more complement. In conducting the antigen titration and complement-fixation tests, two full units are employed, contained in 1 cc. as illustrated below:

Exact unit	0.3 cc.
Full unit	0.35 cc.
Dose (2 full units)	0.7 cc.

Dilution of complement containing 2 full units in 1 cc. $\frac{30}{0.7} = 1:43$

The following table gives additional examples:

Exact unit, cc.	Full unit, cc.	Two full units, cc.	Dilution to use	Preparation
0.3	0.35	0.7	1:43	1 cc. serum + 42 cc. saline
0.25	0.4	0.8	1:37	1 cc. serum + 36 cc. saline
0.4	0.45	0.9	1:33	1 cc. serum + 32 cc. saline
0.45	0.5	1.0	1:30	1 cc. serum + 29 cc. saline

It is always advisable to dilute complement serum with cold saline solution instead of saline kept at room temperature. Complement serum,

and particularly the diluted complement, should always be kept in a refrigerator when not in use.

Occasionally hyperactive complement yields a unit of less than 0.3 cc. of the 1:30 dilution, but when this occurs it is necessary to take arbitrarily

factory and cannot be used.

3. *Titration of Antigen.*—It is not ordinarily necessary to repeat the titrations for hemolytic and anticomplementary units. It is desirable, however, occasionally to titrate for antigenic activity. The following method is recommended.

(a) Prepare a 1:80 dilution of antigen by adding 0.1 cc., drop by drop with shaking between each, to 7.9 cc. of saline solution in a large test tube or small flask. Higher dilutions are then prepared as follows:

4 cc. of 1:80	+ 4 cc. saline solution =	1:160
4 cc. of 1:160	+ 4 cc. saline solution =	1:320
4 cc. of 1:320	+ 4 cc. saline solution =	1:640
4 cc. of 1:640	+ 4 cc. saline solution =	1:1280
4 cc. of 1:1280	+ 4 cc. saline solution =	1:2560

(b) Arrange five rows of test tubes with 6 in each row. In the first tube of each row place 0.5 cc. of antigen 1:80; in the second tube of each row, 0.5 cc. of antigen 1:160; in the third tube of each row, 0.5 cc. of antigen 1:320; in the fourth tube of each row, 0.5 cc. of antigen 1:640; in the fifth, 0.5 cc. of 1:1280; and in the sixth, 0.5 cc. of 1:2560.

(c) Heat 3 cc. of a moderately to strongly positive syphilitic serum in a water bath at 56° C. for fifteen to twenty minutes and prepare 5 dilutions as follows in large test tubes:

1 0 cc. serum + 4 0 cc. saline =	1:5 (0.5 cc. carries 0.1 cc. serum)
0 5 cc. serum + 4 5 cc. saline =	1:10 (0.5 cc. carries 0.05 cc. serum)
0 5 cc. serum + 9 5 cc. saline =	1:20 (0.5 cc. carries 0.025 cc. serum)
2 0 cc. serum 1:20 + 2 cc. saline =	1:40 (0.5 cc. carries 0.0125 cc. serum)
1 0 cc. serum 1:20 + 4 cc. saline =	1:100 (0.5 cc. carries 0.005 cc. serum)

(d) Add 0.5 cc. of the 1:5 serum dilution to each of the 6 tubes of the first row; 0.5 cc. of the 1:10 dilution to each tube of the second row; 0.5 cc. of the 1:20 dilution to each tube of the third row; 0.5 cc. of the 1:40 dilution to each tube of the fourth row; and 0.5 cc. of the 1:100 dilution to each tube of the fifth row.

(e) Add 1 cc. of complement dilution carrying 2 full units to all the tubes.

(f) Set up a serum control carrying 0.5 cc. of 1:5 serum and 1 cc. of complement (2 full units); also, a hemolytic system control carrying 1 cc. of saline solution and 1 cc. of complement (2 full units).

Incubate at 6° to 8° C. for
t 37° C. for ten minutes.

of sheep cells to all tubes.

(i) Mix thoroughly and place in a water bath at 37° C. for one hour; make readings. The serum and hemolytic system controls should show complete hemolysis.

(j) Chart the results as per the following example observed with a strongly positive serum:

Serum in 0.5 cc.	Antigen in 0.5 cc. amounts					
	1:80	1:160	1:320	1:640	1:1280	1:2560
0.005	—	—	++	—	—	—
0.0125	—	+	++++*	++++	++	+
0.025	+	++++	++++	++++	++++	+
0.05	+++	++++	++++	++++	++++	++
0.1	++++	++++	++++	++++	++++	++++

The dilution of antigen to employ in the main tests is the largest amount giving a ++++ reaction with the smallest amount of serum. (Indicated by * in chart in paragraph (j) above.) If three dilutions of antigen give ++++ reactions with the smallest amount of serum, the dose to use should be midway between the highest and lowest.

IV. Procedure for the Test.—1. *Technic of the Test.*—(a) For each serum arrange two test tubes and place 0.5 cc. of saline solution in No. 2 (control). Add 0.2 cc. of heated serum to each tube.

For each spinal fluid arrange two test tubes and place 0.5 cc. of saline solution in No. 2 (control). Add 0.5 cc. of spinal fluid to each tube.

(b) Add 0.5 cc. of diluted antigen carrying the proper dose to the first tubes and mix thoroughly. Allow to stand at room temperature for ten to thirty minutes.

(c) Add 1 cc. of complement (2 full units) to all tubes. In the case of

control containing 0.5 cc. diluted antigen, 0.5 cc. saline solution, and 1 cc. of complement (2 full units). (2) Hemolytic system control containing 1 cc. of saline solution and 1 cc. of diluted complement (2 full units). (3) Sheep cell control containing 2.5 cc. of saline solution. (4) Controls using positive and negative sera are advisable.

TABLE 13.—MAKE-UP OF TYPICAL TESTS

Tube	Serum	Antigen, cc.	Complement (2 full units), cc.	Hemol- ysin (2 units), cc.	Sheep cells (2%), cc.
1	0.2 cc.	0.5	1.0	0.5	0.5
2	0.2 cc. + 0.5 cc. saline	None	1.0	0.5	0.5
<i>Spinal Fluid</i>					
1	0.5 cc.	0.5	1.0	0.5	0.5
2	0.5 cc. + 0.5 cc. saline	None	1.0	0.5	0.5
<i>Controls</i>					
3	Antigen control: 0.5 cc. saline solution	0.5	1.0	0.5	0.5
4	Hemolytic control: 1 cc. saline solution	None	1.0	0.5	0.5
5	Sheep cell control: 2.5 cc. saline solution	None	None	None	0.5

Interval of 10 to 30 min. at room temperature.

Primary incubation in refrigerator at 6° to 8° C. for 15 to 18 hours followed by 10 min. at 37° C.

Secondary incubation in water bath at 37° C.

(e) Mix the contents of each tube by gentle shaking and place in the refrigerator at 6° to 8° C. for fifteen to eighteen hours.

(f) Place tubes in a water bath at 37° C. for ten minutes (not longer).

(g) To all tubes, except the sheep cell control, add 0.5 cc. of hemolysin (carrying 2 units) and to all tubes add 0.5 cc. of 2 per cent sheep cell suspension (well shaken). Mix the contents of each tube by gentle but thorough shaking of the rack and place in a water bath at 37° C. for one hour.

Table 13 shows a typical protocol for the complement-fixation test with serum and spinal fluid:

2. **Reading and Reporting Results.**—Read the degree of inhibition of hemolysis and record for each tube as: — (complete hemolysis); + (25 per cent inhibition recorded as 1); ++ (50 per cent inhibition recorded as 2); +++ (75 per cent inhibition recorded as 3); ++++ (100 per cent inhibition recorded as 4). All serum, antigen, and hemolytic controls should show complete hemolysis. The corpuscle control should show no hemolysis.

The reactions should be reported as: Positive (++++, +++, or ++); doubtful (+ or ±); negative (—). If the serum control, containing no antigen, shows no hemolysis, the serum is anticomplementary and should be reported as such. A second specimen should be obtained in such cases.

V. **Analysis of Difficulties.**—1. **Due to Complement.**—In the great majority of instances difficulties occurring are due to the complement. Occasionally it is too low in hemolytic activity. Sometimes the complement is apparently satisfactory on titration but yet defective in the tests because it is supersensitive to what may be termed the anticomplementary effects of antigen or serum or spinal fluid, and particularly in summation. For this reason it is recommended that egg albumin be used in all spinal fluid tests. Egg albumin, however, is not required in the tests on serum, although it may be added to the antigen control.

2. **Due to Hemolysin.**—This is usually the first reagent suspected, but least likely to be a cause of trouble, especially if it has been previously used with success. If the saline solution and complement are satisfactory, a good hemolysin rarely causes difficulty even after shipment over long distances.

3. **Due to Corpuscles.**—When blood is obtained from an abattoir one sooner or later encounters specimens which have an increased resistance to hemolysis. Such blood must be discarded.

4. **Due to Antigen.**—If no mistakes have occurred in dilution and dosage, this is rarely a cause of trouble. When the antigen control shows incomplete hemolysis it is usually due to some component of the hemolytic system, especially the complement.

5. **Due to Anticomplementary Sera and Spinal Fluids.**—Sera and spinal fluids may be found to be anticomplementary, as shown by incomplete or no hemolysis in the controls. After experience has been gained some of the partial reactions may be read with safety as doubtful or negative, but as a general rule it is safer and wiser to repeat the tests with fresh serum or spinal fluid.

Tests sometimes have to be repeated, and for this reason the unused portions of all sera and spinal fluids should be routinely kept in a refrigerator until the tests are completed.

✓ FALSE POSITIVE SERO-DIAGNOSTIC REACTIONS

Since the sero-diagnostic tests are not truly specific for syphilis, the clinician must acquaint himself with those conditions other than syphilis

which may produce false positive reactions. The false positive reactions which occur with the various complement-fixation and flocculation tests are either technical or biological.

I. Technical False Positive Reactions.—These may be due to some error introduced by the physician in the collection or labeling of the sera, by the secretary or nurse in copying and recording the results of the various tests and by the serologist or technologist in laboratory technic. Such positive results are not usually confirmed when a second specimen is retested by the same or a different procedure. In well regulated laboratories these technical errors are fortunately infrequent. Since this type of false positive reaction may occur even in the best of laboratories, a diagnosis of syphilis should never be made on the basis of a positive blood reaction on a single specimen.

Technical false positive reactions may occur in sera containing no reagin and are more likely to occur with bacterially contaminated bloods or badly hemolyzed specimens. Glassware which is dirty or inadequately or improperly cleansed may also give rise to false positive reactions. Inexact measurements of the various materials employed in the respective tests are also important factors. Furthermore, if the antigen, other ingredients of the antigen emulsion, or any of the three components of the hemolytic system utilized in a serologic test are unsatisfactory because of faulty

II. Biologic False Positive Reactions.—These are due to the presence of a non-specific substance or possibly reagin in serum or spinal fluid, produced by a number of conditions other than syphilis. Any febrile condition or severe metabolic disturbance may a sero-diagnostic tests for syphilis. Non-occur in leprosy, malaria, and infectious mononucleosis, scarlet fever, upper lobe pneumonia, and lymphoma.

Fortunately these conditions usually have other diagnostic characteristics due to them rarely cause false positive reactions. Furthermore, the serologic reactions in these diseases are usually of very low titer, giving rise to doubtful or weakly positive reactions. It is necessary to differentiate between biologic reactions occurring

and the differentiation of true positive (syphilitic) from the false positive (biologic, non-syphilitic) serologic reactions. The better known and more recent of these procedures are the tests devised by Dr. R. L. Kahn and the spirochetal complement-fixation test devised by Dr. Harry Eagle. At the present time, however, none of these methods has proved of sufficient value for adoption as a routine procedure.

PART II

Chemistry

CHAPTER XIII

GENERAL CHEMICAL TECHNIC

By CLEON J. GENTZKOW and WALTER C. TOBIE

It is neither practicable nor desirable in a work of this kind to review all of the principles of quantitative technic, but it is deemed advisable to present here certain methods, procedures and principles of wide application in clinical chemistry.

Quantitative analyses are done to determine either the relative amount of some constituent in a compound or mixture, or the absolute amount in a known quantity of material.

The most commonly used methods are:

1. **Gravimetric Analysis.**—In this method the constituent sought is separated from solution as an insoluble compound of known chemical composition, dried and weighed. From the weight of this new compound,

3. **Colorimetric Methods.**—Here the substance to be determined is made to react chemically so as to produce a colored compound. The intensity or depth of color is then measured by comparison with a standard. It is essential that the depth of color produced be proportional to the concentration of the substances sought. This subject is treated more fully in Chapter XVI.

4. **Gasometric Methods.**—By these methods the particular unknown to be measured is made to react to produce a gas. The volume of gas produced is then measured under standard conditions of temperature and pressure, or the volume may be held constant and the pressure measured in a manometer. The Van Slyke and Neill manometric apparatus, while not an item of standard Medical Department equipment, is used in many of the larger Army laboratories. Gasometric methods are not included in this volume. For further information on this and other special methods and their applications, see the references listed at the end of this chapter.

GRAVIMETRIC METHODS

1. **The Chemical Balance.**—The commonest type of chemical balance consists essentially of a horizontal lever (beam) with two arms of equal length, with a point of support above the center of gravity. The beam rests on a knife-edge as a fulcrum, while the pans pivot on knife-edges at the ends of the arms.

1. **Types of Laboratory Balances**—In all weighing, it is essential that a proper type of balance be used. The balance should be sufficiently accurate for the work required, but no more so. Thus if 10 gm. of a salt is required with an accuracy only to the nearest 0.1 gm. weighing should be done on trip scales. It would be an obvious waste of time and effort to weigh it with great care on a fine analytical balance to the nearest milligram or tenth of a milligram.

(a) *Trip Scales*.—This is a rough form of balance used in weighings to 1 or 2 kg. which do not have to be extremely accurate. It is sometimes called a "pan balance." Since the balance is usually graduated to 0.1 gm. it is better designated as a decigram balance. The common form consists of two platforms, with a graduated scale on the front of the beam, and one or more sliding weights or riders. Free weights to put on the pans are usually provided.

This is a very useful type of balance and is perhaps used more than any other. As handled in many laboratories, particularly in balancing tubes for centrifugalizing, it is subjected to such gross abuse that it soon becomes extremely inaccurate. But if used properly and kept free from dirt and corrosion, it will have a long useful life. In particular, the beam should not be allowed to bang over from one side to another, since this will rapidly ruin the knife edges. Padding the pan stops with rubber will minimize this type of damage. Solid matter should not be allowed to fall on the central knife edge.

(b) *Centigram Balance*.—This type of balance is used in work which must be accurate only to the nearest hundredth of a gram. It usually has a single pan with counterweights on a beam. The smallest scale division for the rider is 0.01 gm. and the maximum capacity is usually about 100 gm.

(c) *Fine Analytical Balance*.—This type of balance is used in ordinary analytical chemical work where weighings are made to the nearest milligram or tenth of a milligram. It should have a capacity of 100 to 200 gm. with a sensitivity of 0.1 mg. when fully loaded. Some of the newer balances, such as the Chainomatic, have devices which eliminate the use of fractional weights and greatly expedite weighings. Micro- and semimicro-balances are further refinements and permit weighing small amounts of material with an accuracy of 0.001 mg. in some cases.

2. **Use and Care of Balances**.—Well-designed and properly constructed balances will give many years of useful service if cared for properly. If neglected or mistreated, they will rapidly become inaccurate and unsatisfactory. The precautions which follow refer mainly to the analytical balance but should also be followed, as far as applicable, with the other types.

(a) The balance should stand on a firm horizontal surface free of vibration. It should not be near a radiator or other source of heat, nor should direct sunlight fall on it, since errors in weighing owing to unequal expansion of the metal will result. The balance must be level, as shown by the spirit level or plumb bob in the case. The pointer should remain at zero when the beam is raised without load, and also when the beam is lowered. The pointer should swing equal distances to either side of the zero when the beam is swinging with no load on the pans. Adjustment of swings is made by the balancing screws on the ends of the beam. When not in use, the

balance case should be kept closed to exclude dust, and the beam should always be raised off the knife edges to prevent injury by jarring.

(b) Always lower the beam slowly and carefully. Do not let the beam rest on the knife edges while weights or materials are being placed on, or removed from, the pans.

(c) The beam is best set in motion by lowering the rider upon it or removing it for an instant. Never start motion by touching the pan or by suddenly lowering the beam upon the knife edge.

(d) Substances to be weighed should never be placed directly upon the pans, but upon watch glasses, weighing papers, or other containers.

(e) Objects to be weighed should be nearly at room temperature, since air currents from a hot body, or condensation of moisture on a cold one, will introduce serious errors.

(f) Never handle weights with the hands or place them on the pans except with the forceps. Substances to be weighed are placed on the left hand pan and the weights on the right hand one.

(g) The pans and the weights must be kept free of dust at all times. For cleaning them, a camel's-hair brush is essential. After several weeks or months, dust will eventually accumulate on the beam and on the agate bearings of the central knife edge. It can usually be removed by careful dusting with the brush. If not, the pans and beam are removed with great care and are gently cleaned with a dry soft cloth such as a freshly laundered handkerchief; a towel is too coarse. After cleaning the balance is readjusted to swing equally without load.

3. *Methods of Weighing.*—(a) *Ordinary Method of Direct Weighing.*—Place the object to be weighed on the left pan. With the forceps, place a weight, judged to be slightly heavier than required, on the right pan. Gently lower the beam a little and note which way the pointer swings; if to the left, the weight is too heavy, if to the right, the object is the heavier. Raise the beam and replace the weight with the next lighter one, if it is found to be too heavy. After the weight to the nearest gram has been determined, continue the process using the fractional weights, gently releasing the pan support each time to see how the pointer swings. Close the balance case and adjust the rider until the pointer swings equal distances to the right and left.

Make all weighings methodically by trying the weights one after another in proper order, after first checking to see that the balance is properly adjusted. Final adjustments and weighings should always be made with the balance case closed to prevent errors due to air currents. Check the final weighing by (1) adding up the weights missing from the box, in which every weight should always have its proper place, and (2) adding up the weights that are on the pan. As a final check, add the weights as they are returned to the box.

In making weighings, there is a tendency to make the pointer swing through too great an arc. A swing of 2 or 3 scale divisions to right and left is generally ample.

(b) *Method of Single Swings.*—Adjust the balance so that the pointer swings 2 or 3 divisions to the right when the pan support is carefully released, and continues to oscillate to the right of the zero. In weighing, add weights until this same deflection is obtained upon releasing the pan support. This method of weighing is very rapid, but not quite as accurate as

the ordinary method. It is not applicable to a balance having a single release operating both beam and balance supports.

(c) *Determination of Sensitivity and Method of Swings.*—In this method, which is very precise, the zero reading of the balance, without any load, must be determined first. Set the beam in motion and record the turning-points, or extreme positions, of the pointer for an uneven number of swings, usually 5, and take the mean of the readings. The first two swings are inaccurate because of the jar in shutting the balance case, and for other reasons, and are disregarded.

For example, suppose the swings are: Left 6.5, right 2.5, left 6.3, right 2.3, left 6.1. The mean of the three left swings would be $\frac{6.5 + 6.3 + 6.1}{3}$

or 6.3; of the two right swings $\frac{2.5 + 2.3}{2}$ or 2.4; and the rest point would be 6.3—2.4 or 3.9 scale divisions to the left. It is customary to give the minus sign to displacements to the left and the plus sign to those to the right, since these signs indicate whether the observed difference is finally to be subtracted from or added to the weight as given by the weights and rider.

The zero reading may change during the course of the day and should be determined before each weighing. When a series of weighings are to be made, determine the zero point at the beginning and at the end of the series and use the mean.

Next determine the sensitivity of the balance for the particular load to be weighed by placing the object on the left pan and the weights on the right until equilibrium is established as nearly as possible. Determine the rest point on the scale in the same manner as in making the zero reading. Add or remove 1 mg. by means of the rider and determine the rest point again. The difference between this and the previous point of rest gives the sensitivity of the balance; that is, the number of scale divisions equal to 1 mg. in weight with the particular load in question.

Assuming that the first point of rest lies at 4.1 scale divisions to the right with a load of 25.723 gm. and the second rest point, with a load of 1 mg. less, or 25.722 gm., at 1.9 scale divisions to the right, then the sensitivity of the balance will amount to 4.1—1.9 or 2.2 scale divisions. Since the zero reading was 3.9 scale divisions to the left and the rest point with a load of 25.723 gm. was at 4.1 divisions to the right, it follows that the object was heavier than the weights in the pan by an amount sufficient to displace the rest point from 3.9 to the left to 4.1 to the right, or 8 scale divisions to the right. The weight equal to this amount of displacement can be calculated from the sensitivity as determined above. Since 2.2 scale divisions correspond to 1 mg., then 8 scale divisions would be $\frac{8.0}{2.2}$ or 3.64 mg., approximately 3.6 mg. The true weight of the object, therefore, would be 25.723 + 0.0036 or 25.7266 gm. The weight is expressed only to the fourth decimal place, since most analytical balances will scarcely detect with certainty less than 0.1 mg.

For the methods of double or transposition weighing, reduction of weighings in air to in vacuo, and for the calibration of weights, reference should be made to the various books on quantitative analysis listed at the end of this chapter.

II. Filtration.—The purpose of filtration is to separate a solid from the liquid in which it is suspended, therefore the pores or interstices of the filter must be smaller than the particles to be retained. Precipitates vary greatly in particle size and in the physical characteristics of the particles, therefore proper selection of a paper or other filtering agent is essential.

1. Paper Filters.—Filter paper varies greatly in diameter, thickness, and porosity, depending upon the use to which it is to be applied. *Qualitative* filter paper is the most common sort, and is used in ordinary filtrations. *Quantitative* filter paper is acid-washed to remove as much mineral matter as possible, so that very little ash is dissolved out when acids are filtered through, or remains behind after combustion of a precipitate on the paper in gravimetric analysis. *Hardened* filter papers are intended to resist pressure when suction is applied, as on a Buchner funnel.

The desirable qualities in filter papers for quantitative work are strength, uniform texture, proper porosity, and low ash. For qualitative work strength and proper porosity are required. Select the paper best suited to the particular work to be done. All catalogs of laboratory supply houses give the characteristics of the various filter papers handled by them and in each package of Whatman filter papers is a user's guide indicating the correct paper for each purpose.

An example of improper selection is the use of ordinary paper for filtering trichloroacetic-acid-precipitated blood serum in calcium determinations, since ordinary papers contain considerable calcium which is dissolved out by the acid. In this case only an acid-washed paper is suitable. On the other hand a blood filtrate for nitrogen determinations should not be filtered through an acid-washed paper. In the manufacture of these papers the acid is neutralized with ammonia and some ammonium salts remain in the paper. The blood filtrate dissolves this out, giving a falsely high reading for nitrogen.

(a) *Gravity Filtration.*—For gravity filtration with conical funnels, a round filter paper is simply folded over to crease it across one diameter. Then without opening the paper, another fold is made at right angles to the first fold. When opened up, the paper should fit the funnel snugly. If it does not, owing to some variation in the angle of the funnel, it should

a paper, considerable time in filtration may be saved by folding the paper so that the liquid passes through it more rapidly. The following method accomplishes this purpose:

- (1) Fold the paper evenly across one diameter.
- (2) Open up the paper and make a second fold at right angles to the first, creasing the paper on the same side.
- (3) Open and turn the paper over, then make a third fold exactly bisecting two of the quarters, creasing on the opposite side from the first two creases.
- (4) Make a fourth fold at right angles to the last one. The paper is now divided into eighths, each segment 45 degrees of the circle, with two creases on one side and two on the other.
- (5) Fold again on the same side as the last, dividing two opposite eighths equally.

(6) Make a final fold at right angles to the previous one, and on the same side. In making the folds be sure all creases pass through the same center point and protect this point with a finger as the creases are made to prevent tearing it.

(7) As the paper is picked up it will tend to shape itself into a cone. Adjust three of the folds, reserving the fourth one to make the paper fit the funnel exactly. This folding gives alternate triple and single thicknesses, with half of the funnel area covered singly. It makes for very rapid

degrees, and a long stem ground at an angle. Such a funnel will give very rapid filtration with a well-fitted filter paper. The stem should fill with a column of filtrate, the weight of this column exerting a suction pull. If much air sucks down the stem past the filter paper, the rate of filtration is usually decreased.

(b) *Suction Filtration*.—For filtration by suction, a Buchner funnel is usually used. It is made of porcelain with a perforated disk to support a filter paper. The stem end of the funnel, fitted with a rubber stopper or other device to make an airtight joint, is fixed in a filtering flask. A hardened filter paper of proper size is placed on the perforated disk, and a slight suction is started, preferably after the paper has been moistened with some of the liquid to be filtered. When the paper has flattened down due to suction, more of the liquid is poured in, and the suction increased to the desired rate. The paper must lie flat on the perforated disk or liquid will suck under the edge of the paper, giving imperfect filtration. While hardened filters are usually employed in suction filtration other types can be used in an emergency. If the suction breaks holes in the paper, use a different grade or use two thicknesses. If no Büchner funnel is at hand, suction filtration can be done by folding a hardened filter to fit a conical funnel.

after filtering
contaminate
such as that
given on page 355, is recommended.

2. *Crucible*
is sha
Put a
and apply gentle suction. Pour enough asbestos suspension into the crucible to form a mat 1 to 2 mm. in thickness. If perforated porcelain disks are available it is best to place one upon the bottom layer of asbestos, then pour a little more asbestos suspension into the crucible with suction. If the disks are not available, it is advisable to use a single mat of asbestos about 3 mm. thick. Wash the mat thoroughly but gently with water until no more asbestos fibers run through. For ordinary filtrations dry the crucible at 105° C., cool in a desiccator over CaCl_2 and weigh. If the precipitate is to be ignited the crucible and mat should also be ignited at the same temperature, cooled and weighed.

3. *Fritted Glass Filters*.—These filters are made by fusing a disk of sintered glass (prepared by partial fusion of ground or powdered glass) into a glass funnel. Many shapes and sizes are available, with the porosity

varying from very fine to very coarse. These filters are particularly useful with solutions which attack filter paper. The most common type is used in place of the Büchner funnel. Other smaller types are used in place of Gooch crucibles avoiding the errors in weighing which sometimes occur when a little of the asbestos is washed through.

These filters must be cleaned *immediately* after filtering alkaline solutions, since retained alkali may attack the disk and alter its porosity. For this reason they should not be used for filtering hot concentrated alkali. If organic liquids have been filtered, the best method of cleaning is to suck large amounts of water through the filter, followed by nitric acid to oxidize any traces of organic matter, then washing thoroughly again to remove the last traces of acid. If a dark coloration persists in the disk, owing to carbonization of organic matter, clean the filter by placing in concentrated sulfuric acid to which about 10 per cent by volume of nitric acid has been added, and gradually heating under a hood until the dark color disappears.

III. Washing Precipitates.—The object of washing a precipitate is to remove as much as possible of the liquid containing dissolved material, while redissolving as little as possible of the precipitate. To do this, the methods of washing must be modified according to the precipitate being handled. All possible cases cannot be considered but the following general principles apply in most instances:

1. In quantitative work on precipitates the size of the filter used should be such that the amount of liquid to be collected on a small filter is not more than half the amount of liquid to be collected on a large filter.
2. The filter should be of such a size as to hold the precipitate without overflowing, while permitting the use of a sufficient amount of washing liquid. In general, the precipitate should not fill the filter more than half full.
3. The liquid should never completely fill the filter, and the upper edge of the filter must be carefully washed with a stream from the wash bottle.
4. To save time, and to prevent redissolving any of the precipitate since no precipitate is *completely* insoluble, the amount of washing liquid should be as small as possible. Amorphous and gelatinous precipitates need more washing than crystalline or granular ones.
5. Let the washing liquid drain as completely as possible before adding a fresh portion.
6. If permissible, use hot water instead of cold water since it passes through the filter more rapidly.
7. It is usually desirable to test the last of the washings to make sure that all soluble matter has been removed.

IV. Weighing Precipitates.—**1. Drying Precipitates.**—Before a precipitate can be weighed, it must be dried. If precipitated on a Gooch crucible or similar apparatus, the usual practice is to heat it to 105° C., in an oven for thirty to sixty minutes, cool to room temperature in a desiccator, then weigh. This is the simplest method of determining the weight of a precipitate, and is preferable, whenever practicable, to the methods which follow.

2. Dry Ignition of Precipitates.—This method in which the precipitate is separated from the filter, the filter burned by itself in a loop of platinum wire, the ash added to the main part of the precipitate and the mixture

then ignited to constant weight, is used in those cases where the ignited substances will be reduced by the burning paper, viz., precipitates of silver chloride, lead sulfate, bismuth oxide, etc. This method is less used than formerly. For details, see the references at the end of the chapter.

3. *Wet Ignition of Precipitates.*—Precipitates which are not altered by the products of combustion of paper, may be ignited wet. Put the filter and precipitate in a crucible, with the paper folded so that the precipitate is not exposed, and so that moisture will be expelled through a layer of paper and not directly to the air, to prevent loss by spattering. Incline the crucible on a triangle, with the cover inclined against the upper edge of the crucible and resting upon the triangle. Direct a gentle flame against the cover. This dries, then carbonizes, and finally burns the filter. Then move the flame to the back of the crucible and slowly increase the temperature until the crucible is subjected to the full heat of the burner. Cool the crucible in a desiccator, then weigh.

TITRIMETRIC METHODS

I. *Volumetric Glassware.*—Pipets, burets, and volumetric flasks are used for accurately measuring liquids by volume. Graduated cylinders are used for less exact measurements. The bell-shaped graduates used in pharmacies are not accurate enough for use in the chemical laboratory.

Pipets, burets, and volumetric flasks are commercially available in four different grades. The "student" or educational grade is the least accurate. Next comes the "retested" grade, which is sufficiently accurate for ordinary quantitative work. The "precision" grade supposedly fulfills the requirements of the U. S. Bureau of Standards, but each item is not tested, and certificates of accuracy are not furnished. The highest grade of glassware available is "certified," meaning that each piece has been tested by the Bureau of Standards and found to comply with its specifications, then etched with the official certification stamp. A certificate is furnished with each piece.

II. *Use of Volumetric Glassware.*—It is absolutely essential that all volumetric glassware be properly cleansed. Traces of oil or grease on the surface of the glass make drops of solution cling to the walls with consequent incomplete emptying or drainage. This holding back of drops of solution introduces serious errors into determinations, especially in the case of pipets. When only a thin film of liquid remains on the surface after draining, the glassware is satisfactorily clean, but the presence of drops indicates grease and dirt. For cleaning methods, see page 354.

1. *Pipets.*—Pipets are essentially tubes, narrowed at either end, and adapted to contain or to deliver a definite amount of liquid, which has been drawn in by suction up to a definite mark, and retained by closing the upper end.

The Ostwald-Folin type of pipet, with a relatively large oval bulb and a short delivery tip, is usually calibrated by the manufacturer to deliver the fixed quantity by blowing out the last few drops remaining in the delivery tip. Such pipets are marked "TD" (to deliver), or sometimes merely "delivers" and are etched with a single or double broad band near the top. With such Ostwald-Folin pipets, *always* blow out the last few drops.

Other bulb pipets (occasionally including some of the Ostwald type) are sometimes marked "TC" (to contain). In exact work they must be rinsed out thoroughly with the diluting solution used in the particular analysis.

Transfer pipets, with a cylindrical bulb approximately in the middle, are calibrated to deliver a fixed volume. *Never blow out this type of pipet*, but allow it to drain freely, then bring the lower tip of the pipet in contact with the surface of the liquid in the receiver and gently draw it up the wet wall, holding it there for a few seconds to allow as much liquid as will run out, to do so.

Mohr type pipets are graduated to deliver a fixed volume and also decimal fractions of this volume. Such pipets should be blown out *if graduated to the tip*, but the final graduations of the tip are frequently inaccurate, so that the graduations on the taper of the tip should not be used if a number of portions of liquid are being measured out of one filling. In using other portions of the pipet, control the outflow with the finger to permit the walls to drain.

2. Burets.—Burets are rather similar to graduated (Mohr) pipets, since the greater part of the cylindrical portion is graduated into subdivisions. Instead of being held in the hand, burets are fixed in a vertical position to a ringstand or similar support, and draining is controlled by a stopcock or pinchcock at the bottom, the top end being open to the atmosphere. In reading, the lowest point of the meniscus is noted. The eye must be at this same level. Burets should always drain cleanly. If they do not, pour the bore full of some form of warm cleaning fluid (see p. 355), and allow to stand for thirty minutes or longer, drain, rinse with distilled water, and test for completeness of draining.

The glass stopcocks of burets require special care. They must be kept clean and lubricated to prevent "freezing." For lubricants see page 355. In emergencies petroleum jelly ("vaseline") can be used. Use a minimum of lubricant or the bore of the stopcock may become obstructed. Remove old lubricant before applying fresh. Commercial lubricants such as "Lubrisal" are convenient and good.

Solutions of alkalis *should not* be allowed to stand overnight in burets with glass stopcocks, since the latter may "freeze." Rinse such burets carefully with distilled water after use with alkali. For methods of removing frozen stopcocks, see page 356. For measuring strong alkalis the buret without stopcock is frequently used. Attach a short length of rubber tubing and a short glass tube drawn out to a point to the buret tip. Insert a glass tube into the rubber tubing, and by means of gentle pressure at the end of the glass tube, the liquid may run out. Make sure that no entrapped air bubbles are discharged, since this will give an error in the volume of liquid delivered. Instead of the glass bead, an ordinary spring pinchcock is sometimes used.

III. Calibration of Glassware.—As supplied by the Army Medical Department, the following are the standard conditions for the calibration of glassware:

1. **Volumetric Flasks.**—Cleanse and dry the flask, then weigh it on a consideration the
 to 1 part per 1000;
 to 1 gm. Instead
 of weighing the flask, it may be counterpoised with shot. Calculate the weight of water necessary to fill the flask to the mark at the proper temperature from Table 14 and place this weight on the balance pan. Fill the flask with distilled water, which has been freshly boiled and cooled, until balance is attained, making sure that no drops of water adhere to the neck of the flask. Mark the lowest point of the meniscus with a wax pencil sharpened to a chisel edge, then etch in the mark or make a scratch mark with a diamond pencil or sharp file.

TABLE 14 — APPARENT WEIGHTS AND VOLUMES OF WATER WEIGHED IN AIR
 (For use in calibration of volumetric apparatus)

Temp., °C.	Wt. of 1 cc	Vol. of 1 gm.	Temp., °C.	Wt. of 1 cc	Vol. of 1 gm.
15	0.9979	1.0021	23	0.9966	1.0034
16	0.9978	1.0022	24	0.9964	1.0036
17	0.9977	1.0023	25	0.9961	1.0039
18	0.9975	1.0025	26	0.9959	1.0041
19	0.9973	1.0027	27	0.9956	1.0044
20	0.9972	1.0028	28	0.9954	1.0046
21	0.9970	1.0030	29	0.9951	1.0049
22	0.9968	1.0032	30	0.9948	1.0052

NOTE The figures in this table, from Landolt and Börnstein's "Tabellen," are based on the weights of water per cubic centimeter which must be weighed into a glass vessel under ordinary conditions in order to indicate the mark to which the vessel must be filled with freshly boiled and cooled distilled water so that it will contain the desired volume at 20° C. Corrections for the buoyant effect of air upon vessel and weights are included.

2 **Pipets.**—(a) *For Delivery.*—Weigh the water delivered into a weighing bottle containing a layer of paraffin oil a few millimeters thick to prevent evaporation. If the original mark is not accurate, make another with a wax pencil and test the pipet again. Continue until the mark is located correctly, then etch or mark with a file.

The weight of 1 cc. of water at any temperature from 15° to 30° C. may be found in Table 14.

(b) *For Uncalibrated or Repaired Pipets.*—Transfer and Ostwald pipets with broken delivery tips may often be repaired but must then be recalibrated. Make a mark on the stem a definite distance, such as 50 mm., above the old calibration mark, using a chisel-edged wax pencil. Weigh the water delivered when the pipet is filled to each mark. From the difference in the two weights, the weight of water contained in each millimeter length of the stem can be calculated. Place the new calibration mark the correct distance above the old one and test by weighing the water delivered. If correct etch in the new mark.

Example: The weights of the water delivered at 20° C. by a 10-cc pipet from two preliminary marks 50 mm. apart are 9.900 and 10.275 gm., respectively. Hence the weight of a column of water between marks is 0.375 gm. or $\frac{0.375}{50} = 0.0075$ gm. per millimeter of column length. The weight of 10 cc. of water at 20° C. is 9.972 gm. or 0.072 gm. more than that delivered from the lower preliminary mark. Hence the correct mark is $\frac{0.072}{0.0075} = 9.6$ mm. above the lower mark.

(c) *Calibration of Pipets to Contain.*—Fill the pipet with water from a weighing bottle which has been weighed before and after. From the weight of water contained, calculate the volume from the data in Table 14.

3. *Burets.*—To calibrate a buret of 25- or 50-cc. capacity, fill it with water to a point slightly above the zero mark. Run out the water slowly until the bottom of the meniscus just touches the mark. Wait a minute for complete drainage of the walls to take place. Adjust the meniscus again to the zero point if necessary. Remove excess water from the tip of the buret. Then run a 2-cc. portion into a weighing bottle containing a little liquid petrolatum, bottle and petrolatum having first been weighed. After delivering the 2-cc. portion, touch the drop adhering to the tip to the surface of the petrolatum carefully so as to remove it. Weigh the bottle and contents. Continue with 2-cc. portions over the entire range of the buret, then take the temperature of the water.

Multiply the weight in grams of each portion of water by the volume of 1 gm. at the observed temperature, as taken from Table 14, in order to calculate the actual volume delivered. From the actual volume delivered, make a table of corrections.

IV. *Indicators.*—Indicators are compounds, usually organic, which indicate the pH of a liquid by means of the color which they give. The colors, or changes in color, are ascribed to specific arrangements or rearrangements in the molecule of the indicator. The chemistry of the color changes is complex, and will not be considered here.

Although the distinction is not a sharp one, indicators may be roughly classed into two types: (a) Titration or endpoint indicators which give a rather sudden shift of color at some particular pH. Such indicators are discussed in this chapter. The second type (b), consists of indicators which undergo a rather gradual and uniform alteration in color over a considerable range of pH, within which range they are useful in determining the pH of solutions. They are discussed under Hydrogen Ion Concentration, Chapter XIV.

1. *Phenolphthalein.*—This is probably the most commonly useful indicator in titrations. It is suitable for titrating organic and inorganic acids with strong bases, or vice versa, but does not give a clear endpoint in the presence of ammonia or ammonium salts. The free CO_2 in ordinary distilled water causes an appreciable error, particularly if 0.1 or 0.01 *N* acids and alkalis are used. In titrating from the acid side, it goes from colorless to pink at about pH 8.2. The pink deepens as the pH increases, but at about pH 10 the color disappears again. This must be remembered if very strongly alkaline solutions are involved. The indicator is usually used in 1 per cent solution. Dissolve 0.5 gm. in 50 cc. of 95 per cent alcohol, dilute to 50 cc. with water, filter if necessary, and place in a (4) cc. dropping bottle. Use 1 to 4 drops per 100 cc. of solution to be titrated.

2. *Methyl Orange (Tropaeolin D).*—In alkaline solution this indicator is yellow, changing to red on the addition of a strong acid, but not with a weak acid. Hence this indicator is suitable for titrating strong mineral acids in the presence of carbonic acid or weak organic acids. In titrating alkaline solutions with strong acids, the color is yellow down to pH 4.4, then changes through orange and orange-red, to red at pH 3.1 and lower. The indicator solution contains 0.1 g. of methyl orange in 100 cc. of distilled water. Use 5 drops for each 100 cc. of solution to be titrated,

since if too little indicator is used, the red color will not appear in acid solution, probably owing to hydrolysis of the indicator in the presence of excess water. It requires a certain degree of practice and experience to obtain satisfactory titrations with methyl orange.

3. **Methyl Red.**—This indicator is useful in titrating weak organic bases and ammonia. It is not very sensitive to carbonic acid, but is more so than methyl orange, so that it is less suitable for the titration of carbonates. In alkaline solutions it gives a very pale yellow down to pH 6.2 shifting to violet-red at pH 4.2 and below. Titrating with a strong acid such as HCl, the shift from yellow to red is very abrupt, no intermediate color being noticeable. The indicator solution contains 0.02 gm. of methyl red dissolved in 100 cc. of hot water and filtered before use. Add 2 to 3 drops for each 100 cc. of solution titrated.

4. **Other Indicators.**—Various other titration indicators are used for special purposes. For a summary of the characteristics of the more common titration indicators, see Table 15.

TABLE 15—INDICATORS

Common name	Chemical name	pH range	Color		Preparation of indicator solution	Principal uses in titration
			Acid	Alkaline		
Topfer's reagent	p-Dimethylaminazobenzene	2-9.4	Red	Yellow	0.5% alcoholic	Free HCl in gastric contents
Methyl orange	Sodium dimethylaminoazobenzene sulfonate	3-1-4	Red	Yellow	0.02-0.1% aqueous	Mineral acids and strong bases, mineral acids in presence of carbonic acid
Congo red	Sodium tetrasodiphenyl-naphthosulfonate	3-5	Blue	Red	0.5% alcoholic	Titration of weak bases and ammonia by mineral acids
Methyl red	o-Carboxybenzenesulfonamethylamine	4-2-6	Red	Yellow	0.02% aqueous	Titration of weak bases and ammonia
Aloxan red	Sodium aloxan monosulfonate	4-5	Yellow	Purple	1% aqueous	Total acidity of gastric contents except combined HCl
Nitrazine paper	Sodium dimetaphenyl-azonaphthol disulfonate	4-5-7	Yellow	Blue	Paper or solution	Approximate pH urine
Litmus paper		4-5-8	Red	Blue	Paper	Reaction of urine, reaction of various solutions
Phenolphthalein		8-3-10	Colorless	Red	0.1-1% in 50% alcohol	Titration of strong acids and bases, organic acids by strong bases

V. **Standard Volumetric Solutions.**—1. **Definitions.**—(a) **Molar Solutions.**—A molar solution of a chemical compound is a solution containing 1 gram-molecule of the compound in 1 liter of solution. The molecular weight in grams of the compound is dissolved in water or other solvent and made up to 1 liter. A gram-molecule is also known as a "mole." For example, the molecular weight of sodium chloride is 58.454, so a molar solution contains 58.454 gm. (one mole) of NaCl in 1 liter of solution. The molecular weight of sulfuric acid is 98.076, so a molar solution (abbreviated as "1 M" or "M") contains that weight in grams of absolute H₂SO₄ per liter of solution.

(b) **Normal Solution.**—A normal solution of a compound contains 1 gram-equivalent of the reactive constituent of the compound in 1 liter of solution. By gram-equivalent is meant the amount of substance chemically equivalent to 1 gram-atom (1.008 gm.) of hydrogen. Stated differently, a normal (abbreviated as "N" or "1 N") solution is one which contains 1 gm.-atom of reacting hydrogen per liter of solution, or which can quantitatively replace, or react, directly, or indirectly, with an equal volume of such a solution.

(1) **Normal Acid Solutions.**—In acid-alkali titration, a normal acid solution contains per liter the amount of acid which has 1 gm.-atom of

both senses of the term, since it combines with oxygen to form water, reducing its charge of $+1$ to 0 .

(c) *Precipitation and Substitution Reactions.*—In these reactions, accu-

(d) *General Precautions.*—In titrimetric analysis, it is important to eliminate sources of error as far as possible. When filling a buret for the first time, particularly after cleaning, rinse 3 times with about 10 cc. of the standard solution to be used, draining the buret completely each time. Invert or shake bottles or flasks containing standard solutions before pouring into burets, to rinse any condensed moisture off the walls. In any given determination or series of determinations, titration should always be to the same endpoint, i. e., accurate results would not be obtained if the same acid and alkali were titrated to colorless with phenolphthalein on one occasion and to a light pink on another.

In preparing many standardized solutions, the use of carbon dioxide-free water is essential or desirable. To prepare it, place about 1 liter of distilled water in a 2-liter Erlenmeyer flask, boil gently for about five minutes, then discontinue heating, and cover the flask mouth with an inverted beaker. When cooled to room temperature, stopper the flask with a clean rubber stopper.

For accurate work as in determining the normality of solutions, all titrations should be done in duplicate and the average result used for computations. If the two determinations do not check run others until checks are obtained.

(e) *Calculations.*—The fundamental mathematical expression in titrations and other volumetric reactions is:

$$N' \times \text{Volume}' = N'' \times \text{Volume}'' \text{ or, } N' \times \text{cc.}' = N'' \times \text{cc.}''$$

In other words, 100 cc. of an alkali which is 1 N will neutralize 50 cc. of an acid which is 2 N. The volume of one solution used, multiplied by its own normality equals the volume of the other solution times its normality. It is preferable to make up a solution for standardization which is stronger than desired so that exact normality can be obtained by diluting.

VI. Preparation of Standard Solutions.—A. Acidimetry and Alkalimetry.
—1. Standard Hydrochloric Acid ($\text{HCl} = 36.46$).

(a) *Standard HCl by Constant Boiling Mixture Method.*—The following method is that of Hulett and Bonner, modified by the use of the formula of W. H. King.* It depends upon the fact that when hydrochloric acid solution is distilled, the concentration of acid in the distillate approaches a constant concentration which depends upon the barometric pressure.

To concentrated hydrochloric acid (sp. gr. 1.19 to 1.20, 35 to 37 per cent HCl) add an equal volume of water. Bring the specific gravity to 1.1 at 25°C . by adding more water or more acid. Distil the mixture continuously at a rate of 5 to 10 cc. per minute, from a distilling flask attached to a condenser having a straight inner tube. Apparatus with ground-glass joints is preferable. If not available, use rubber stoppers in preference to cork, which is attacked by HCl . It is best to distil large volumes, 1 to 1.5 liters

* Jour. Assn. Off. Agr. Chemists, 25, 653, 1912.

of mixture may be distilled in a 2-liter flask. To prevent superheating which causes variation in the concentration of HCl in the distillate, put several small pieces of glass tubing in the distilling flask. Electrical heating gives the best results. If a flame is used for heating, the flask should be protected with sheet asbestos so that hot gases from the flame strike the flask only at the bottom. After three-fourths of the mixture has been distilled, change receivers and collect the distillate, which is now constant boiling, until only about 10 per cent of the original volume remains in the flask.

Read and correct the barometer at the end of the distillation. For most accurate results correct the barometer reading to 0° C., by referring to a table (see Reference No. 4 at the end of the chapter). If such a table is not available, an *approximate* correction at temperatures of 20° to 30° C., and pressures of 740 to 780 mm. of mercury may be made by deducting 2.50 mm. from the observed height of mercury in the barometer.

The constant-boiling mixture should be preserved in a tight glass-stoppered bottle, labeled with the date, the barometric pressure at which it was distilled, etc. It will keep indefinitely. The first three-fourths of the distillate should be saved for future preparations of constant-boiling acid.

Calculate the weight in grams of constant-boiling HCl (G) required to give one equivalent weight of HCl (36.4648) from the following equation:

$$G = \frac{P + 7680}{46.8356}$$

where P is the barometric pressure of Hg corrected to 0° C. This formula is applicable to pressures of 540 to 780 mm. of Hg. In this range it is accurate to 1 part in 1000. At pressures approximating 760 mm. of Hg, the accuracy is 1 part in 10,000.

A 0.1000 N standard solution is prepared by diluting the proper *weight* (not volume) of the acid calculated from the formula to 1 liter with CO₂-free water. Pipet 16.1 cc. (approximately 18 gm.) of the constant-boiling mixture into a 50-cc flask, preferably glass-stoppered to protect the balance. More is added or withdrawn by means of a medicine dropper with a fine drawn-out tip, until the exact weight is obtained. The acid is then diluted with CO₂-free water, rinsed into a 1-liter volumetric flask, and diluted to volume at 20° C. Further standardization is unnecessary.

(b) *Standardizing Hydrochloric Acid against Crystalline Calcium Carbonate.*—Dilute pure concentrated hydrochloric acid with 11 volumes of CO₂-free water. This gives a solution which is slightly more than 1 N. Add a known volume (say 50 or 100 cc.) of the diluted acid to a weighed crystal of Iceland spar (calcite, crystalline calcium carbonate). Cover the beaker or flask with a watch glass. When the evolution of CO₂ has entirely ceased, remove the crystal, wash in distilled water, dry, and reweigh. Then,

$$20 \times \frac{\text{gm. of CaCO}_3 \text{ dissolved}}{\text{cc. of acid used}} = \text{normality of HCl ("factor")}$$

If a given volume of acid somewhat stronger than 1 N is diluted to factor times the volume, the diluted acid will be exactly 1.000 N. Thus if the normality (factor) is found to be 1.046, for example, 91 cc. of CO₂-free water added to a volume of 100 cc. and mixed well will give 191 cc. of a solution which is exactly 1.000 N.

both senses of the term, since it combines with oxygen to form water, reducing its charge of $+1$ to 0 .

(c) *Precipitation and Substitution Reactions.*—In these reactions, accurate determinations are made by precipitating or substituting by means of a standardized solution. In determining silver in alloys, the silver is precipitated as the chloride (AgCl) from a solution of the alloy in nitric acid, NaCl solution of known concentration being used as the precipitant.

(d) *General Precautions.*—In titrimetric analysis, it is important to eliminate sources of error as far as possible. When filling a buret for the first time, particularly after cleaning, rinse 3 times with about 10 cc. of the standard solution to be used, draining the buret completely each time. Invert or shake bottles or flasks containing standard solutions before pouring into burets, to rinse any condensed moisture off the walls. In any given determination or series of determinations, titration should always be to the same endpoint, *i. e.*, accurate results would not be obtained if the same acid and alkali were titrated to colorless with phenolphthalein on one occasion and to a light pink on another.

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For accurate work as in determining the normality of solutions, all titrations should be done in duplicate and the average result used for computations. If the two determinations do not check run others until checks are obtained.

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normality so that exact normality can be obtained by diluting.

VI. Preparation of Standard Solutions.—A. Acidimetry and Alkalimetry.

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(b) *Against Acid Potassium Phthalate.*— $\text{KHC}_8\text{H}_4\text{O}_4 = 204.14$. In many laboratories it is customary to use acid potassium phthalate (potassium hydrogen phthalate, potassium biphthalate) as the primary standard. The best quality is that furnished by the U. S. Bureau of Standards, but the grade prepared by commercial manufacturers for standardizing alkali is nearly as satisfactory. Let the phthalate stand overnight in a Petri dish in a desiccator over technical CaCl_2 to remove traces of moisture. Normal solutions cannot be prepared since the KH phthalate is insufficiently soluble in water. To make a 0.1000 N solution, weigh out exactly 20.414 gm., and make this to a volume of 1 liter with CO_2 -free water. Standardize NaOH against this solution using phenolphthalein. The standardized NaOH solution can then be used to standardize HCl or other acid solutions. The 0.1000 N phthalate solution will keep for several months in a refrigerator, but if evidences of mold growth appear, discard the solution.

4. *Other Standard Alkali Solutions.*—Standardized KOH solutions are prepared by a method analogous to that for NaOH. In preparing standardized barium hydroxide solutions, CO_2 -free distilled water need not be used, since some insoluble bicarbonate is always present in any case and must be removed by filtration before standardizing. Remember that a normal solution of $\text{Ba}(\text{OH})_2$ is half molar. In standardizing sodium carbonate solutions, use methyl orange indicator.

TABLE 16—TEMPERATURE CORRECTION PER 1000 CC. FOR NORMAL VOLUMETRIC SOLUTIONS FOR REDUCTION OF VOLUME TO STANDARD TEMPERATURE OF 20° C.

Temperature	Water and 0.1 N solution	1 N HCl	1 N H_2SO_4	1 N $\text{H}_2\text{C}_2\text{O}_4$	1 N KOH and NaOH	1 N Na_2CO_3
15	+0.77	+0.97	+1.30	+1.05	+1.33	+1.29
16	+0.64	+0.79	+1.06	+0.85	+1.08	+1.05
17	+0.49	+0.61	+0.81	+0.65	+0.82	+0.80
18	+0.34	+0.41	+0.55	+0.44	+0.55	+0.54
19	+0.17	+0.21	+0.28	+0.23	+0.28	+0.27
20	0	0	0	0	0	0
21	-0.19	-0.22	-0.28	-0.24	-0.29	-0.28
22	-0.39	-0.44	-0.56	-0.49	-0.59	-0.56
23	-0.60	-0.67	-0.85	-0.75	-0.90	-0.85
24	-0.81	-0.91	-1.15	-1.02	-1.21	-1.15
25	-1.04	-1.17	-1.46	-1.29	-1.52	-1.46
26	-1.28	-1.43	-1.78	-1.57	-1.84	-1.77
27	-1.53	-1.70	-2.11	-1.85	-2.17	-2.09
28	-1.80	-1.98	-2.45	-2.04	-2.50	-2.41
29	-2.05	-2.26	-2.79	-2.44	-2.84	-2.75
30	-2.33	-2.55	-3.13	-2.77	-3.19	-3.09

NOTE. The figures for water and 0.1 N solutions are applicable to all solutions 0.1N or weaker and also to 1 N solutions of sodium chloride.

Example (1) In a titration 46.15 cc. of N HCl were used at a temperature of 23° C. At the standard temperature of 20° C., this amount of solution would occupy a volume of—

$$46.15 - \frac{46.15 \times 0.67}{1000} = 46.12$$

From the table, the volume of the solution is, therefore, 46.12 cc. in a full liter at standard temperature. This number is the factor by which the actual number of cc. of solution used in a titration must be multiplied by this factor, the result will be the number of cc. of an exactly 0.1 N solution.

(3) A liter flask is calibrated to hold exactly that volume at 20° C. A normal solution of NaOH is prepared at 25° C. The table shows that the solution at 25° C. would occupy at 20° C. exactly 1.52 cc. less, so in order to make the solution exactly normal, 1.52 cc. of water should be added.

5 Volume Corrections for Standard Solutions.—In preparing all standard solutions and in making all measurements in volumetric flasks, the solution should be at the same temperature at which the flask was calibrated. Minor variations in temperature introduce only slight error. For the most exacting type of work corrections as shown in Table 16 must be applied.

B. Oxidation and Reduction Reactions.—1. Standard Potassium Permanganate Solutions.— $\text{KMnO}_4 = 158.03$. If used as an oxidizing agent in acid solution (see page 153), one molecule of KMnO_4 will oxidize 5 atoms of H or the equivalent. Therefore, a 0.1 N solution will contain 1/50th of a mole of the salt, or 3.161 gm. in 1 liter. A solution can be made with fair accuracy by directly weighing out and dissolving this amount of crystals. However, the solution tends to become weaker by reduction of traces of organic matter in the distilled water, liberating MnO_2 which further catalyzes the decomposition so that the solution rapidly loses strength upon storage.

Add 3.2 gm. of KMnO_4 to a liter of distilled water in an Erlenmeyer flask and boil gently for ten to fifteen minutes. Cover the flask with a beaker. After standing overnight, filter the solution through asbestos on a Gooch crucible, or better yet, a firm layer of asbestos on a plug of glass wool in a conical funnel, filtering by gravity.

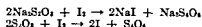
Dissolve a carefully weighed amount, 0.25 to 0.3 gm. of pure sodium oxalate previously dried for an hour at 105°C. , and cooled in a desiccator over CaCl_2 , in 200 to 250 cc. of hot water (80°C. or higher) in a 600-cc. beaker. Cautiously add 5 cc. of concentrated sulfuric acid. Titrate with the KMnO_4 solution, stirring the liquid vigorously and continuously with a thermometer, holding the temperature above 60°C. Add about 40 cc. of the KMnO_4 over a period of about two minutes. Add the last of the KMnO_4 solution slowly, allowing each drop to become decolorized before the next one is added. The endpoint is taken when a faint pink color persists for thirty seconds.

$$\text{Normality of } \text{KMnO}_4 = \frac{\text{gm. of sodium oxalate}}{\text{cc. of } \text{KMnO}_4} \times 0.067$$

The value of 0.067 is the milli-equivalent of Na oxalate

If the normality of the KMnO_4 solution is near 0.1 N, it is commonly used as it is, and the 0.1 N equivalent calculated by multiplying the cc. used in any titration by the normality factor. It is best preserved in a

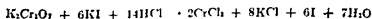
accurate, but are of wide applicability. The fundamental reaction is the conversion of sodium thiosulfate and free iodine to sodium tetrathionate and sodium iodide.



The method can be used not only to determine free iodine, but also
The indicator used is
1 free iodine, the color

1. **Starch Indicator Solution** In 50 cc. of boiling water add 1 gm. of soluble starch, stirring with a glass rod until solution is complete, then dilute to 100 cc. with cold water. Starch indicator solutions often deteriorate by becoming moldy after a few days. This change may be prevented or retarded by dissolving 1 gm. of salicylic acid in the water used in preparing the solution. In the absence of soluble starch, an indicator solution can be made by adding 1 gm. of ordinary cornstarch to 100 cc. of water, and gradually bringing to a boil, while stirring vigorously to prevent burning. A clear sensitive solution may also be prepared by extracting the breakfast food "Puffed Rice" with water.

2. **Potassium Dichromate, 0.1 N** $K_2Cr_2O_7 = 294.20$. This salt liberates iodine from iodide according to the equation:



Since one mole of the dichromate oxidizes or liberates 6 atoms of I from KI, and each atom of I is equivalent to one of hydrogen, a liter of normal solution of $K_2Cr_2O_7$ contains one-sixth of a mole and a tenth normal solution one-sixtieth of a mole. Therefore, dissolve 4.903 gm. of pure $K_2Cr_2O_7$ (dried in a desiccator if the atmosphere is damp) in distilled water and make to a volume of 1 liter in a volumetric flask. This standard solution is stable for several months at room temperature in a well-closed container.

3. **Sodium Thiosulfate, 0.1 N.**— $Na_2S_2O_4 \cdot 5H_2O = 248.20$. Dissolve 26 gm. of Na thiosulfate in 1 liter of distilled water which need not be CO_2 -free. Pipet exactly 25 cc. of 0.1 N potassium dichromate into a 600-cc. beaker containing 50 cc. of water, 10 cc. of concentrated HCl, and 3 gm. of KI (weighed only to the nearest 0.1 gm.). A brown color is produced by the liberation of I. Cover the beaker, let it stand in the dark for five minutes for the reaction to proceed, then add 300 cc. of water and titrate with the Na thiosulfate solution. When the brown color has faded, add about 3 drops of starch indicator solution, and titrate to the disappearance of the blue iodo-starch color.

$$\text{Normality of thiosulfate} = \frac{\text{cc. of dichromate solution}}{\text{cc. of thiosulfate solution}}$$

... listed to exactly 0.1000 N. ... it may be preferable not ... is required. Thiosulfate solutions frequently undergo gradual decomposition, with the liberation of free sulfur and a reduction in normality. This decomposition can be greatly delayed, and a standardized solution preserved for about six months, if about 10 cc. of 0.1 N NaOH is added at the time the thiosulfate is dissolved and made to 1 liter.

4. **Iodine Solution, 0.1 N.**— $I = 126.92$. In a glass container on the trip scales, weigh out 13 gm. of solid iodine. Dissolve 30 gm. of KI in about 250 cc. of water. In a liter cylinder or volumetric flask, mix the iodine with the KI solution until it is completely dissolved, then make up to 1 liter with distilled water. Standardize by titrating 25 cc. with standardized thiosulfate solution, using 3 drops of starch indicator solution toward the end.

$$\text{Normality of iodine solution} = \frac{\text{cc. of thiosulfate} \times \text{normality}}{\text{cc. of iodine solution}}$$

FLUOROMETRY

Fluorescence is the property possessed by certain substances of emitting radiation of a different wave length, usually a longer one, than that which falls upon them. Thus certain substances, usually in solution, may be quantitatively determined by measuring the amount of visible light which they emit when invisible ultraviolet light shines upon them. The method has been increasingly used in recent years, particularly in the determination of vitamins, atabrine and quinine, and other substances in low concentrations. A similar procedure is also applicable to the qualitative detection of certain substances in the dry state. Since only a few specialized Army laboratories are at present equipped for fluorometry, details of the methods will not be given.

CHROMATOGRAPHY

Chromatography, sometimes known as the chromatographic analysis of Tswett, from the Russian botanist who devised the method, is a procedure in which a solution containing two or more dissolved substances is drawn through a column of some adsorptive solid which is insoluble in the solvent used, for the purpose of adsorbing the dissolved substances upon the insoluble material comprising the column. With a proper choice of solvent and of adsorbent, the dissolved substances will adsorb on the solid in different portions of the column, depending upon differences in their chemical structure. They may thus be separated, and purified by appropriate treatment.

The method has been of increasing importance in recent years, particularly in the separation of the complex mixtures of pigments which occur in plants and in animal tissues and body fluids. It may also be used for the separation of colorless substances. The use of chromatography permits the separation of substances which could not readily be isolated in any other fashion. The subject is too extensive to be considered in detail here. For technics and applications of the method, see references Nos. 5 and 6 at the end of the chapter.

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CHAPTER XIV

HYDROGEN ION DETERMINATION

By WILLIAM MANSFIELD CLARK

THEORETICAL CONSIDERATIONS

I. Hydrogen Ion Concentration, Activity, and pH.—Originally pH was defined as the logarithm of the reciprocal of the hydrogen ion concentration of an aqueous solution. With the advance of theory it has become evident that the values actually measured, and still called pH numbers, do not conform strictly to this definition. The reasons for this, which are given in numerous treatises, need not be reviewed here, for we may arrive quickly at a practical definition the interpretation of which shows the significance of pH.

The basic device for determining a pH number is the hydrogen cell. This furnishes a means of comparing two solutions with respect to the states of their available protons. Being fundamentally a means of *comparison* the device makes it necessary that ultimately one of the solutions in a series be a primary standard. Since we shall assume here that the hydrogen cell will not be used in routine measurements, we may assume that a satisfactory primary standard was provided for the evaluation of those secondary standards which will be specified as necessary to methods here described. When such a standard (of pH = 0) is placed in one-half cell of the hydrogen cell and the secondary standard is in the other half-cell the following relation holds, subject to a small, uncertain, and usually neglected error due to the inadequacy of the customary method of dealing with liquid junction potentials.

$$pH = \frac{EF}{2.3026 RT}$$

E is the potential difference between the electrodes, the electrode in the tested solution being negative to that in the standard solution when the pH value of the tested solution is greater than zero. F is the faraday. The number 2.3026 is the modulus for converting Napierian logarithms to logarithms of base 10. R is the gas constant and T is the absolute temperature. At 25° C., for example, the relation is

$$pH = \frac{E}{0.05912}$$

The theory at the basis of this relation reveals the advantage of using pH numbers. A pH number is in linear relation to the potential of the hydrogen cell, one half-cell of which contains the primary standard. E , in turn, is in linear relation to the difference between the free energy per equivalent of protons as they exist in the tested solution and the free energy per equivalent of protons as they exist in the primary standard. It is this relative energetic quantity, together with the equally relative constant characteristic of a particular system of proton donor (*e. g.*, acid)

and conjugate proton acceptor (*e. g.*, acid anion) which determines the state of that system. For example, consider the pH of the blood which is 7.4. According to the old definition of pH we were inclined to place emphasis on the calculated hydrogen ion concentration which is only about 0.000,000,04 normal. A quantity at this concentration can have little physical effect in and of itself. What has always counted is the use of such a "calculation value" to determine the ratio of the considerable concentrations of each acid and its corresponding anion. What is also emphasized anew is less the absolute values of these *concentrations* than the more direct relation of pH to what may be called, in non-technical language, effective concentrations, or, more properly, "activities."

Attention may well be called to another aspect. Frequently the solutions studied for biological purposes are too complex for detailed analysis; or else there is no occasion to make the detailed analysis. For example, the growth of a bacillus may depend upon the states of the foodstuffs, enzymes and tissue components which are proton donors or acceptors. Although the parts played by each of these may be unknown, there still may be established empirically a correlation between growth and the pH number of the culture medium. Such empirical studies, which dodge the detail of particular relations among sets of specific proton donors and receptors, legitimately employ pH as the more direct measure of the general state of affairs with respect to equilibria among acids, bases and their salts.

If the hydrogen atom be given the symbol H, its nucleus, the *proton*, may be designated by H^+ . In water solution there are doubtless no "free" protons but those which have escaped from acids are combined with water, so that the hydrogen ion in water solutions is H_3O^+ .

A few examples of proton donors and the corresponding proton acceptors, examples which represent different types, are shown in Table 17. In the third column are the values of pK' the number of which corresponds to the pH value at which the ratio of the concentration of the proton acceptor to that of the corresponding proton donor is approximately unity.

For details consult reference 1 at the end of this chapter.

TABLE 17

Proton donors	Proton acceptors	pK' (approx)
H_3O^+ (hydrogen ion)	H_2O (water, simplest molecule)	
H_2O (water)	OH^- (hydroxyl ion)	
HAc (acetic acid)	Ac^- (acetate anion)	4.7
$H_2PO_4^-$ (primary phosphate anion)	HPO_4^{2-} (secondary phosphate anion)	6.8
NH_4^+ (ammonium cation)	NH_3 (ammonia)	9.4
$CH_2NH_3^+$	$CH_2NH_2^+$	
(glycine cation)	(glycine dipole ion)	2.4
COOH	COO^-	
$CH_2NH_3^+$	CH_2NH_2	
(glycine dipole ion)	(glycine anion)	9.8
COO^-	COO^-	

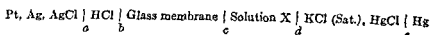
II. Buffer Action.—A mixture of a weak acid and its alkali salt, a mixture of an amino acid and its sodium salt or hydrochloride, proteins, and, indeed, a great variety of materials when in water solution exhibit the or alkali pH than i is added solutions

exhibiting the effect are called buffer solutions. For a mixture of a simple acid and its salt the effect is maximal at a pH value corresponding to the pK' value and falls off gradually on either side, becoming practically useless at about 1.5 units of pH greater or less than the value of pK' . The effect is also, of course, a function of the concentration of the buffer system.

Buffer solutions have innumerable uses. We shall confine attention to their use as the practical, secondary standards for the following methods of determining pH numbers.

METHODS

1. The Glass Electrode.—Of the several designs of cells containing a glass membrane the following is the more common.



If the above scheme be read with construction, we have the follow there is a difference of potential.

and this in turn is coated with silver chloride. When this electrode is immersed in a definite solution of hydrochloric acid there is a difference of potential at a between electrode and solution, determined by the concentration of chloride. This silver : silver chloride electrode is usually within a Solution

the glass membrane there is a difference of potential $b + c$, which, for a good glass membrane, is in linear relation to the difference between the pH number of the standard hydrochloric acid solution and the pH number of solution X. To complete the cell, liquid junction is made with a saturated KCl solution which in turn is in contact with mercury overlaid with calomel, the electrode potential being e . If we assume that the use of a saturated solution of KCl reduces the liquid junction potential at d to a small and negligible value, all potential differences are constant except that across the glass membrane and this is in linear relation to the pH value of the unknown solution X under test. First the system is standardized by using in succession several secondary standards which are the buffer solutions of Table 19. Each is placed on the outside of the glass bulb. The relation between the pH numbers and the over-all potentials of the cell is usually linear, at constant temperature, between about pH 1 and 9. A chart showing the relation permits the reading of the pH number of the tested solution when the potential of the cell has been measured with the tested solution at the position "solution x." This method of comparison with standards and interpolation is preferable to reliance on a theoretical equation for the cell.

Since the resistance of the glass membrane is usually higher than that which will permit the use of an ordinary galvanometer as null point instrument in the potentiometric measurement of the cell, the more common instruments make use of the electron tube in null-point detectors. There are many designs of instruments used for this purpose and some of them represent the latest improvements in engineering. The directions, which accompany the better instruments, should be followed strictly.

The ease and rapidity with which measurements may be made often

lead an operator to a false sense of their precision. Obviously the precision depends in the first instance on the care taken to make the standard buffer solutions meet specifications and upon the good technic with which they are used. Both glass electrode and potentiometric instrument are subject to idiosyncrasies which sometimes require good technical knowledge to trace. There are also a number of well-known sources of error for the discussion of which the reader should consult reference 2 at the end of this chapter. Particular attention should be given to electrical leakage over damp surfaces especially in humid climates.

II. Acid-base Indicators.—An acid-base indicator may be defined for present purposes as a substance which, in aqueous solution, exhibits unique absorption of radiant energy and which, on gain or loss of a proton, exhibits another unique absorption of radiant energy. For visual observation of a color-change which occurs with change of pH, at least one of the species should absorb in the visible part of the spectrum.

Reliable indicators obey the law:

$$\text{pH} = \text{pK}' + \log \frac{\text{concentration of species 2}}{\text{concentration of species 1}}$$

where species 2 is that which contains one less proton than species 1. The above relation indicates that insofar as but one color-transformation is concerned (see Table 18) each indicator is limited in the range of pH within which it shows appreciable color-change. Hence, in the use of indicators to test the pH value of an unknown it is necessary to make a preliminary trial in order to select that indicator which will be partially transformed in the tested solution.

III. Colorimetric Method with Standards.—One method of use is to compare the color of the tested solution with the colors of buffer solution standards in accordance with the following specifications.

It is assumed that tested solution and buffer solution have the same pH number when their colors match under the following conditions:

(a) The buffer solution standard and the tested solution must contain the same indicator at the same concentration, usually 0.5 cc. of indicator to 10 cc. of solution.

(b) Each solution must be viewed through the same length of absorbing column (tubes of equal bore) and the incident light must be of the same intensity in both cases.

Caution.—In using standards supplied in sets, use only the indicator solution furnished with them since it is the same as that used for the preparation of the colored standards. For the unknown use only tubes of the same bore as those containing the standards.

If the unknown be colored to begin with, surprisingly good measurements may be made with the aid of a block comparator constructed as follows:

Parallel to one another and very close together, holes are bored in a block of wood as indicated by the circles of Figure 10. The holes should be only slightly larger than the tubes to be used. For viewing pairs of tubes in tandem, bore holes perpendicular to the axes of each of the first pair of holes. Blacken all exposed surfaces of the wood with a non-reflecting stain. Place the unknown plus the indicator in the position shown and back it by a water blank. In the other two positions place colored stand-

ards, backing up each with an untreated sample of the unknown to compensate for its natural color. Change the buffer standard until a color match is made with the unknown. Interpolate the color intermediate between the two standards.

The primary assumption that two solutions have the same pH number when their colors match under the conditions specified is not always adequate. The standard and the solution under test may have the same color and yet differ appreciably in pH number, as determined by the hydrogen cell, if one solution differs much from the other in temperature, total salt concentration or concentration of a substance which combines with the indicator. For the corrections which then must be made consult advanced texts.

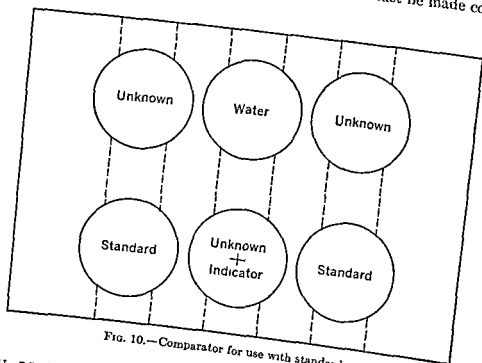


Fig. 10.—Comparator for use with standards

IV. Method Without Standards.—For stations where it would be inconvenient to supply the somewhat elaborate series of standard buffer solutions the following method is useful.

As shown by the equation, page 164, the pH number of a solution may be calculated if the pK' value of the indicator used be known (see Table 18) and the ratio of the concentrations of the two species of that indicator in the tested solution can be determined. The principle on which the ratio is determined experimentally is as follows: When 10 drops of indicator are in 10 cc of the tested solution imagine the indicator equivalent to x drops to be in Form 2 and that equivalent to $10 - x$ drops to be in Form 1. The color of this combination can be reproduced as follows: To water, which is made alkaline enough to transform the indicator practically completely to Form 2, add x drops of indicator and dilute to 10 cc. To another tube of water, which is made acid enough to convert the indicator practically completely to Form 1, add $10 - x$ drops and dilute to 10 cc. When these two solutions are in tubes of like bore and are viewed

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$$pH = -\log_{10} [H^+]$$

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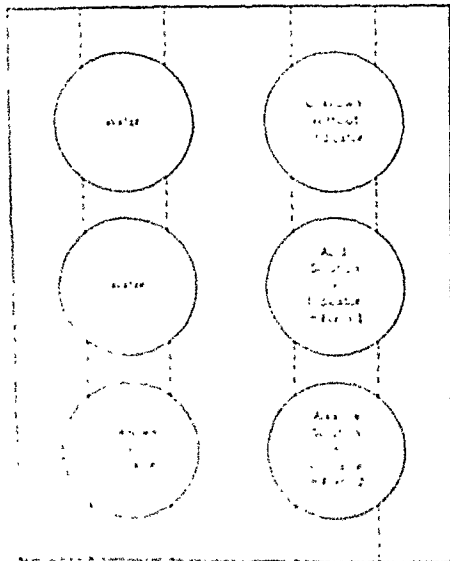


Diagram illustrating the relationship between Acid and Base in the context of the Genius Differentiation.

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0.3 cc., etc. If the depth of color produced by 1 cc. of indicator solution is too great, 0.5 cc. may be used, in which case x becomes 0.05, 0.10, 0.15, cc., etc.

It usually suffices to judge the complete conversion of the indicator to Forms 1 and 2 merely by observation on addition of dilute acid for the first case and dilute alkali for the second case. But since several of the indicators listed in Table 18 undergo a change to a red form in very acid solution (which may be used for the measurement of low pH values) care must be taken that the acidification is not too intense.

When this method is used with a colored solution the comparator block of Figure 11 permits compensation. If the unknown with indicator is placed in the front, left-hand position as shown, water blanks should be placed in the two holes back of it. The front, right-hand hole contains the solution with the indicator completely transformed to Form 2, the hole directly back of it, the solution with the indicator completely transformed to Form 1 and the last hole the unknown without indicator.

V. Use of Indicators in Titrations.—The second principal use of an indicator is to determine the so-called end-point, or equivalence point, in a titration. When a solution of an acid is titrated with a standard solution of an alkali, or when a solution of a base is titrated with a standard solution of a strong acid, equality in the equivalents of acid and base is characterized by a pH number which depends upon several factors, principally the pK' value of the titrated system and the dilution.

In many common cases the pH which is characteristic of this equivalence point need not be determined with great precision because near the equivalence point a very large change of pH occurs on the addition of a very small quantity of acid or alkali and in consequence a small error in detecting the proper pH number will make a negligible error in the titer. By the same token there is chosen for end-point detection an indicator which will exhibit a distinct color change within the permissible limits for the end-point pH.

For example, in the titration of 0.1 M acetic acid by 0.1 M sodium hydroxide the ideal end-point is about pH 8.6. An error of about 1.4 units pH in either direction will involve an error of about 0.1 per cent. Since phenolphthalein at a well chosen, high dilution will be practically colorless at pH 8.0 and will be distinctly pink at pH 8.6, considerably less than the above error will be made by stopping the titration on the appearance of a distinct and permanent pink color.

Since the pH number for an end-point is a function of the pK' value for each acid and the dilution, it is impracticable to specify any general simple rule. If the end-point for a particular titration is not given with the specific directions of the analytical method where the titration is specified, the approximate value may be found in the charts of Chapter XXVIII of the *Determination of Hydrogen Ions*—Clark. (Reference 1 at the end of this Chapter.)

VI. Indicators. Table 18 lists by common names the more frequently used indicators. For the purpose of the method given on page 165, which does not require prepared standards, it is necessary to know the value of pK' . This corresponds to the pH value of the half-transformation of the species giving the color listed first to the species giving the color listed last. The values of pK' are approximate and are valid only for 25° C. and for solutions having salt concentrations near those of the buffer standards.

The pH ranges listed in the third column are purely arbitrary and are given only for convenience.

Litmus (azolitmin) is an indicator of variable composition and is not reliable for precise measurements. Its pH range is roughly: red 4.5-8.3 blue. Litmus paper is convenient for noting whether a solution is distinctly acid or alkaline. Indicators formerly in common use but which are not recommended are Congo-red (blue 3.0-5.0 red), alizarine red S (yellow 3.7-4.2 pink), alizarine (yellow 5.5-6.8 red; violet 10.1-12.1 purple), neutral red (red 6.8-8.0 yellow).

Some indicators, such as litmus and neutral red, are reduced by bacterial cultures. Reduced neutral red (colorless) undergoes a secondary color-change to yellow that often causes confusion with the yellow form of the oxidant.

If tests of reduction are to be made, the reliable oxidation-reduction indicators, and not those used for pH measurements, should be used.

TABLE 1b — CHARACTERISTICS OF INDICATORS

Name	pH'	pH range and colors	cc 0.01 N NaOH per 0.1 gm. (a)
Thymol blue (acid range)	1.7	red 1.2-2.8 yellow	21.5
Methyl yellow (b)	3.3	red 2.9-4.0 yellow	(c)
Methyl orange (d)	3.5	red 3.1-4.4 yellow	(e)
Bromphenol blue	4.0	yellow 3.1-4.7 blue	14.9
Bromocresol green	4.7	yellow 3.8-5.4 blue	14.3
Methyl red	5.0	red 4.2-6.3 yellow	(f)
Chlorphenol red	6.0	yellow 5.1-6.7 red	23.6
Bromocresol purple (g)	6.2	yellow 5.4-7.0 purple	18.5
Bromthymol blue	7.1	yellow 6.1-7.7 blue	16.0
Phenol red	7.8	yellow 7.0-8.6 red	28.2
Cresol red	8.3	yellow 7.4-9.0 red	26.2
Thymol blue (alkaline range)	8.9	yellow 8.0-9.6 blue	21.5
Phenolphthalein (h)	9.7	colorless 8.2-10.0 red	(i)

(a) To make a 0.04 per cent stock solution of an indicator for which a number is given in the last column grind 0.1 gm. of the pure, acid indicator with the number of cc of 0.01 N NaOH given and when solution is complete dilute to 250 cc with distilled water.

(b) Methyl yellow (Töpfer's indicator) (p-dimethylaminoazobenzene) is included only because clinicians use it in measurements of gastric acidity.

(c) Stock solution of methyl yellow: 0.01 gm. + 0.1 cc 0.1 N HCl + 80 cc 95 per cent ethanol + 20 cc water.

(d) Do not use with phthalate buffers.

(e) Stock solution of methyl orange: 0.05 gm. in 100 cc water.

(f) Stock solution of methyl red: 0.02 gm. in 60 cc 95 per cent ethanol. Add 40 cc. water.

(g) Beware of dichromatic effect when used to determine pH of turbid solution.

(h) Fades in strong alkali.

(i) Stock solution of phenolphthalein: 0.05 gm. in 50 cc. 95 per cent ethanol. Add 50 cc water.

VII. Standard Buffer Solutions.—Although there are several reliable series of standardized buffer solutions, we reproduce here the specifications of those which have been used perhaps the more frequently. The pH numbers of these solutions were determined by means of the hydrogen cell. Their reproduction requires meticulous care in the preparation of the solutions. These are essentially secondary standards and are to serve as the standards for the glass electrode and indicator methods. The weights of materials specified do not always conform to molecular weights calculated

with the most recent values of atomic weights. This is because the international atomic weights were slightly different at the time the measurements were made.

It is essential that properly purified chemicals be used. Most laboratory supply houses now furnish chemicals of special purity which may be used directly for the preparation of standard buffer solutions. Methods of purification of ordinary reagent grade chemicals are given below along with directions for preparing the stock solutions.

The stock solutions required are: M/5 potassium chloride (KCl), M/5 acid potassium phosphate (KH_2PO_4), M/5 acid potassium phthalate ($\text{KHC}_8\text{H}_4\text{O}_4$), M/5 potassium chloride (H_3BO_3 and KCl), and M/5 hydrochloric acid (HCl). These solutions and their subsequent dilution should be redistilled. "Conductivity water" distilled first from acid chromate solution and again from barium hydroxide is recommended but is not absolutely essential.

1. **M/5 Potassium Chloride.**—The salt should be recrystallized three or four times and dried in an oven for seventy-two hours. The M/5 solution contains 14.912 gm. per liter.

2. **M/5 Acid Potassium Phthalate Solution.**—Make up a concentrated solution of potassium hydroxide by dissolving 60 gm. of a high-grade sample in about 400 cc. of distilled water. Add 50 gm. of the commercial *resublimed* anhydride of orthophthalic acid. Test a cooled portion of the solution with phenolphthalein; if still alkaline, add more phthalic anhydride; if acid, add more KOH. When roughly adjusted to a slight pink with phenolphthalein, add as much more phthalic anhydride as the solution contains and heat until all is dissolved. Filter hot, and allow crystallization to take place slowly. Drain the crystals by suction and recrystallize at least twice from distilled water. Crystallization should not be allowed to take place below 20° C. since on chilling crystals of a more acid salt are deposited. Dry the salt at 110° to 115° C. to constant weight. A fifth-molecular solution contains 40.828 gm. per liter.

3. **M/5 Acid Potassium Phosphate Solution.**—Recrystallize a high-grade commercial sample of the salt at least three times from distilled water and dry to a constant weight at 110° to 115° C. A fifth-molecular solution contains 27.232 gm. per liter. The solution should be distinctly red to methyl red and distinctly blue to brom phenol blue.

4. **M/5 Boric Acid—M.5 Potassium Chloride.**—Recrystallize the boric acid several times from distilled water. Dry in the air, as boric acid loses water of constitution above 58° C., to a constant weight. The constancy of weight may be established by drying small portions in a desiccator over calcium chloride. Add purified potassium chloride to the boric acid solution to bring the salt concentration in the borate mixture to a point comparable with that of the phosphate mixtures, so that colorimetric checks may be obtained with the two series where they overlap. One liter of the solution should contain 12.4015 gm. of boric acid and 14.912 gm. of potassium chloride.

5. **M.5 Sodium Hydroxide Solution.**—This solution is the most difficult to prepare since it should be as free as possible from the carbonate. Dissolve 100 gm. of NaOH in 100 cc. of distilled water in a pyrex glass flask.

Cover the mouth of the flask with tinfoil and allow to stand until the carbonate has mostly settled. Decant only the *clear* supernatant fluid and dilute quickly, after rough calculation, to a concentration somewhat greater than 1 N. Withdraw 10 cc. of this dilution and standardize with an acid solution of known strength. From this standardization calculate the dilution required to furnish a M/5 solution. Make the dilution with the least possible exposure to the air and pour the solution into a *paraffined* bottle to which a calibrated 50-cc. buret and a soda-lime guard tube have been attached. The solution must now be most carefully standardized and it is preferable to use a factor rather than attempt adjustment to exactly M/5.

6. M/5 Hydrochloric Acid.—Dilute a high-grade hydrochloric acid solution to about 20 per cent and distil. Dilute the distillate to approximately M/5 and standardize with the M/5 sodium hydroxide made as given above. It is well to standardize carefully by the silver chloride method and check with the standardized alkali.

From these stock solutions mixtures are prepared corresponding to increments of 0.2 pH as shown in Table 19.

TABLE 19.—COMPOSITION OF MIXTURES GIVING pH VALUES AT 20° C. AT INTERVALS OF 0.2

Phthalate-HCl Mixtures

2 2	50 cc. M/5 KHPhtalate	46 60 cc. M/5 HCl	Dilute to 200 cc.
2 4	50 cc. M/5 KHPhtalate	39 60 cc. M/5 HCl	Dilute to 200 cc.
2 6	50 cc. M/5 KHPhtalate	33 00 cc. M/5 HCl	Dilute to 200 cc.
2 8	50 cc. M/5 KHPhtalate	26 50 cc. M/5 HCl	Dilute to 200 cc.
3 0	50 cc. M/5 KHPhtalate	20 40 cc. M/5 HCl	Dilute to 200 cc.
3 2	50 cc. M/5 KHPhtalate	14 50 cc. M/5 HCl	Dilute to 200 cc.
3 4	50 cc. M/5 KHPhtalate	9 95 cc. M/5 HCl	Dilute to 200 cc.
3 6	50 cc. M/5 KHPhtalate	6 00 cc. M/5 HCl	Dilute to 200 cc.
3 8	50 cc. M/5 KHPhtalate	2 65 cc. M/5 HCl	Dilute to 200 cc.

Phthalate-NaOH Mixtures

4 0	50 cc. M/5 KHPhtalate	0 40 cc. M/5 NaOH	Dilute to 200 cc.
4 2	50 cc. M/5 KHPhtalate	3 65 cc. M/5 NaOH	Dilute to 200 cc.
4 4	50 cc. M/5 KHPhtalate	7 35 cc. M/5 NaOH	Dilute to 200 cc.
4 6	50 cc. M/5 KHPhtalate	12 00 cc. M/5 NaOH	Dilute to 200 cc.
4 8	50 cc. M/5 KHPhtalate	17 50 cc. M/5 NaOH	Dilute to 200 cc.
5 0	50 cc. M/5 KHPhtalate	23 65 cc. M/5 NaOH	Dilute to 200 cc.
5 2	50 cc. M/5 KHPhtalate	29 75 cc. M/5 NaOH	Dilute to 200 cc.
5 4	50 cc. M/5 KHPhtalate	35 25 cc. M/5 NaOH	Dilute to 200 cc.
5 6	50 cc. M/5 KHPhtalate	39 70 cc. M/5 NaOH	Dilute to 200 cc.
5 8	50 cc. M/5 KHPhtalate	43 10 cc. M/5 NaOH	Dilute to 200 cc.
6 0	50 cc. M/5 KHPhtalate	45 40 cc. M/5 NaOH	Dilute to 200 cc.
6 2	50 cc. M/5 KHPhtalate	47 00 cc. M/5 NaOH	Dilute to 200 cc.

KH₂PO₄-NaOH Mixtures

5 8	50 cc. M/5 KH ₂ PO ₄	3 66 cc. M/5 NaOH	Dilute to 200 cc.
6 0	50 cc. M/5 KH ₂ PO ₄	5 64 cc. M/5 NaOH	Dilute to 200 cc.
6 2	50 cc. M/5 KH ₂ PO ₄	8 55 cc. M/5 NaOH	Dilute to 200 cc.
6 4	50 cc. M/5 KH ₂ PO ₄	12 60 cc. M/5 NaOH	Dilute to 200 cc.
6 6	50 cc. M/5 KH ₂ PO ₄	17 74 cc. M/5 NaOH	Dilute to 200 cc.
6 8	50 cc. M/5 KH ₂ PO ₄	23 60 cc. M/5 NaOH	Dilute to 200 cc.
7 0	50 cc. M/5 KH ₂ PO ₄	29 54 cc. M/5 NaOH	Dilute to 200 cc.
7 2	50 cc. M/5 KH ₂ PO ₄	34 90 cc. M/5 NaOH	Dilute to 200 cc.
7 4	50 cc. M/5 KH ₂ PO ₄	39 34 cc. M/5 NaOH	Dilute to 200 cc.
7 6	50 cc. M/5 KH ₂ PO ₄	42 74 cc. M/5 NaOH	Dilute to 200 cc.
7 8	50 cc. M/5 KH ₂ PO ₄	45 17 cc. M/5 NaOH	Dilute to 200 cc.
8 0	50 cc. M/5 KH ₂ PO ₄	46 85 cc. M/5 NaOH	Dilute to 200 cc.

TABLE 19 — (Continued)

Boric Acid, KCl, NaOH Mixtures

7 8	50 cc. M/5 H_3BO_3 , M/5 KCl	2 65	cc. M/5 NaOH	Dilute to 200 cc.
8 0	50 cc. M/5 H_3BO_3 , M/5 KCl	4 00	cc. M/5 NaOH	Dilute to 200 cc.
8 2	50 cc. M/5 H_3BO_3 , M/5 KCl	5 90	cc. M/5 NaOH	Dilute to 200 cc.
8 4	50 cc. M/5 H_3BO_3 , M/5 KCl	8 55	cc. M/5 NaOH	Dilute to 200 cc.
8 6	50 cc. M/5 H_3BO_3 , M/5 KCl	12 00	cc. M/5 NaOH	Dilute to 200 cc.
8 8	50 cc. M/5 H_3BO_3 , M/5 KCl	16 40	cc. M/5 NaOH	Dilute to 200 cc.
9 0	50 cc. M/5 H_3BO_3 , M/5 KCl	21 40	cc. M/5 NaOH	Dilute to 200 cc.
9 2	50 cc. M/5 H_3BO_3 , M/5 KCl	26 70	cc. M/5 NaOH	Dilute to 200 cc.
9 4	50 cc. M/5 H_3BO_3 , M/5 KCl	32 00	cc. M/5 NaOH	Dilute to 200 cc.
9 6	50 cc. M/5 H_3BO_3 , M/5 KCl	36 85	cc. M/5 NaOH	Dilute to 200 cc.
9 8	50 cc. M/5 H_3BO_3 , M/5 KCl	40 80	cc. M/5 NaOH	Dilute to 200 cc.
10 0	50 cc. M/5 H_3BO_3 , M/5 KCl	43 90	cc. M/5 NaOH	Dilute to 200 cc.

NOTE.—It is important to check the consistency of any particular set of these mixtures by comparing "5.8" and "6.2 phthalate" with "5.8" and "6.2 phosphate" using bromcresol purple. Also "7.8" and "8.0 phosphate" should be compared with the corresponding borates using cresol red.

If standard buffers outside of the ranges listed are desired, consult reference No. 1 below.

REFERENCES

1. CLARK, W. M.: The Determination of Hydrogen Ions, 3d ed., Baltimore, Williams & Wilkins Company, 1928.
2. DOLE, MALCOLM The Glass Electrode, New York, John Wiley & Son, 1941.

CHAPTER XV

QUANTITATIVE URINALYSIS

By CLEON J. GENTZKOW

QUANTITATIVE determinations of urinary constituents should be made only on representative samples taken from twenty-four-hour specimens, properly preserved and measured. Directions for collection and preservation are given in Chapter I. In general, refrigeration is the best method of preservation. Of the chemical preservatives, toluol (toluene) is quite efficient and produces least interference in subsequent procedures.

I. Albumin.—While none of the simpler methods for albumin determination are absolutely accurate, that given here is sufficiently so for all clinical purposes as shown by repeated checks against more elaborate methods.

Sedimentation Method of Shevky and Stafford.—1. **Reagents.**—(a) *Tsuchiya's Solution.*—Fifteen grams of phosphotungstic acid, 50 cc. concentrated hydrochloric acid and 1000 cc. of 95 per cent alcohol, all mixed.

2. **Procedure.**—The first step is the dilution of the urine. Nephritic urines are usually diluted ten-fold. In urines with very scanty protein content a lower dilution or none at all will give more exact results. Occasionally a urine is encountered with more than 2.8 per cent of protein which is the maximum that can be determined by a ten-fold dilution. In such a case, a fresh sample is diluted twenty-fold and the determination repeated.

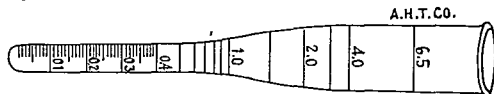


FIG. 12.—Shevky-Stafford tube.

Measure 4 cc. of the diluted urine into a Shevky-Stafford graduated centrifuge tube, the 4-cc. mark on the tube itself serving for the measurement. Add Tsuchiya's reagent to the 6.5-cc. mark, mix by slowly inverting three times, allow to stand for exactly one minute, then centrifugalize for exactly ten minutes at 1800 r.p.m. Read the volume of precipitate on the scale.

3. **Calculation.**—Grams of protein per liter of urine = cc. of precipitate $\times 7.2 \times$ dilution where dilution indicates the number of times the urine was diluted before the sample was measured into the tube.

II. Bence-Jones Protein.—This substance, proteose-like in character, is occasionally found in the urine. It does not react immunologically like any of the normal blood proteins. It is believed to be of some diagnostic importance in cases of multiple myeloma and myelogenous osteosarcoma, and is excreted in the urine of some cases of hyperparathyroidism, leukemia and empyema.

A. Qualitative Test.—1. **Reagents.**—(a) *Acetic acid.*

2. **Procedure.**—Heat 10 cc. of urine to 40° C. for a few minutes; a cloudiness will appear. Gradually heat to about 60° C.; a flocculent precipitate will develop. Acidify slightly with acetic acid and heat to 100° C.; the precipitate will largely disappear. Filter while boiling hot. The precipitate returns on cooling the tube.

This property of precipitating at so low a temperature and dissolving at a higher temperature is typical of Bence-Jones protein and differentiates it qualitatively from all other protein material occurring in the urine.

B. Quantitative Test.—1. **Reagents.**—(a) *Acetic acid.*

(b) *Alcohol, ethyl, 50 per cent.*

2. **Procedure.**—In a tared centrifuge tube place 10 cc. of urine, add just sufficient acetic acid to render it slightly acid and keep overnight in a water bath at 60° C. Centrifugalize, and pour off the supernatant liquid. Add 10 cc. of 50 per cent ethyl alcohol, mix thoroughly and centrifugalize. Pour off the alcoholic supernatant liquid and dry the residue at 100° C. to a constant weight. Cool and weigh. The increase in weight is the Bence-Jones protein present in 10 cc. of urine.

3. **Calculation.**—From the increase in weight determined for a 10-cc. specimen the percentage for the twenty-four hour sample may be calculated.

III. Total Nitrogen.—Approximately 95 per cent of the total nitrogen of the urine comes from the urea, uric acid, creatinine and ammonia. The balance is derived from the small amounts of amino acids, hippuric acid, purine bases, pigments, etc. As ordinarily done for clinical purposes a complete nitrogen partition includes the determination of the total non-protein nitrogen and also the percentages of the total occurring in the form of urea, uric acid, ammonia and creatinine. Of greatest clinical interest are the total nitrogen, the urea nitrogen and the ammonia nitrogen.

Albumin, if present, must be removed. Add 10 per cent acetic acid to 100 cc. of urine in a beaker until distinctly acid to litmus, avoiding any excess. Heat slowly to boiling and boil for two minutes. Filter while hot through a nitrogen-free paper.

If the albumin is to be determined, wash the precipitate thoroughly with hot water, then determine the nitrogen of the paper and precipitate by the Kjeldahl method given below. Calculate the weight of albumin by multiplying the figure for nitrogen by 6.26. Each cc. of 0.1 N acid used in the Kjeldahl titration is equivalent to 1.4 mg. of nitrogen or 8.75 mg. of albumin.

The Kjeldahl method depends upon the conversion of the various nitrogenous substances into ammonium sulfate by boiling with concentrated sulfuric acid, its subsequent decomposition by means of a fixed alkali and the collection of the liberated ammonia in an acid of known strength. The uncombined acid is then titrated with a known strength alkali.

1. **Reagents.**—(a) *Sulfuric acid, concentrated* (specific gravity 1.84).

(b) " " " " " "

(c) " " " " " " (specific gravity 1.50).

(d) *Sulfuric acid, 0.1 N.*

(e) *Sodium hydroxide, 0.1 N.*

(f) *Pumice*

2. **Procedure.**—Place 5 cc. of urine in a 500-cc. pyrex Kjeldahl flask. Add 20 cc. of concentrated sulfuric acid, 0.2 gm. of copper sulfate and boil the mixture for some time after it is colorless or nearly so. Allow the flask

to cool and dilute the contents with about 200 cc. of ammonia-free water. Add a little more of a concentrated solution of sodium hydroxide than is necessary to neutralize the sulfuric acid, using 2 to 3 drops of methyl red solution as an indicator, coarse pumice stone to prevent bumping, and a small piece of paraffin to lessen the tendency to froth. Connect the flask with a condenser so arranged that the delivery tube passes into a receiving flask containing 50 cc. of 0.1 N sulfuric or hydrochloric acid with 5 to 10 drops of methyl red. The delivery tube must reach

order to avoid the sucking back of the acid. Boil until about one-half is distilled over. Should the indicator change toward the alkaline side, more 0.1 N acid must be added. The excess of acid is titrated with 0.1 N sodium hydroxide.

3. *Calculation.*—Subtract the number of cc. of 0.1 N sodium hydroxide used in the titration from the number of cc. of 0.1 N sulfuric acid taken originally. This represents the acid neutralized by the ammonia, each cc. corresponding to 1.4 mg. of nitrogen. Then $\frac{n \times 0.0014 \times 100}{s} = \text{gm. nitrogen per 100 cc. of urine}$ where n = net cc. of 0.1 N acid and s = cc. of urine used.

IV. *Urea Nitrogen (Urea).*—A. *Colorimetric Method* (Gentzkow assisted by Masen).—1. *Reagents.*—Sodium tungstate, sulfuric acid, ammonium sulfate standard, potassium persulfate, potassium gluconate, powdered urease and Nessler's solution as described under Blood Chemistry, page 203, and in addition:

(a) *Acetate Buffer Mixture.*—Dissolve 15 gm. of crystallized sodium acetate in a 100-cc. volumetric flask with 50 to 75 cc. of water. Add 1 cc. of glacial acetic, dilute to volume and mix.

(b) *Permutit.*

2. *Procedure.*—To 5 cc. of urine add about 5 gm. of permutit. Shake gently for three minutes to absorb ammonia, then filter. Dilute 1 cc. of the filtered urine to 50 cc. To 5 cc. of this diluted urine add 34 cc. of water and 1 cc. of the acetate buffer mixture. After mixing, add 10 to 20 mg. of the urease powder, shake and let stand at room temperature which must not be below 20° C. for twenty minutes.

Next add 5 cc. of 10 per cent sodium tungstate solution, mix and then 5 cc. of 0.66 N sulfuric acid. Shake vigorously, let stand a few minutes and filter through a good grade of qualitative paper, such as Whatman No. 2. Pour the filtrate through a second time to insure absolute clarity.

Place 5 cc. of filtrate in a test tube graduated at 20 and 25 cc. In a similar tube place 5 cc. of the working standard ammonium sulfate solution (0.015 gm. N per cc.). Dilute each to the 20-cc. mark.

Prepare the nesslerizing solution by mixing 1 part of the gluconate and 1 part of the persulfate solutions. Pour this mixture into an equal volume of Nessler's solution (1 gluconate, 1 persulfate, 2 Nessler's). This mixture must be used within fifteen minutes.

To the standard and to each unknown add 4 cc. of the mixed reagent, dilute at once to the 25-cc. mark, stopper with paraffined stoppers and shake vigorously. Allow to stand for fifteen minutes to develop full color.

Compare in a visual colorimeter using a Wratten No. 75 filter. With

a photoelectric colorimeter use a filter with maximum transmission at 490 to 500 m μ . (See page 191 for use of the photoelectric colorimeter.)

3. Calculations.—For the visual colorimeter:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 7.5 = \text{gm. urea N per liter of urine}$$

Multiply by 2.14 to give grams urea per liter of urine.

B. Van Slyke and Cullen Aération Method (Modified).—1. Reagents.—

(a) *Urease*, Squibb, double strength, powdered, and

(b) *Acetate buffer mixture*, same as in Method A.

(c) *Potassium carbonate*: Use the dry, granulated salt or a saturated solution of potassium carbonate made by dissolving 90 gm. of potassium carbonate in 100 cc. of distilled water. The sodium salt may be used.

(d) *Sulfuric acid*, 0.02 N.: One cc. reacts exactly with 0.28016 mg. nitrogen or 0.60053 mg. urea.

(e) *Caprylic alcohol*.¹

(f) *Alizarin or methyl red indicator solution*.

2. *Procedure*.—Have ready 5 large tubes, measuring about 32 x 204 mm., holding about 125 cc., with aërating tube, short tube, a 2-hole rubber stopper and rubber tubing connection for each. Mark them "1," "A," "B," "C," and "D" in the order in which the stream of air will pass through. In tube "1" place 20 cc. of approximately 1 N sulfuric acid to remove ammonia from the incoming air; in "A" 10 cc. of ammonia-free water together with 2 drops of the acetate buffer mixture and 50 mg. of urease; in "B" and "D" place 25 cc. 0.02 N acid and 5 drops of the indicator; in "C" place 5 cc. of urine and 5 drops of caprylic alcohol. Insert stoppers tightly, being sure the aërating tubes reach nearly to the bottom of each tube. Remove the stopper from "A" and add 5 drops of caprylic alcohol, exactly 0.5 cc. of urine, holding the pipet tip down near the liquid already in the tube, and stopper the tube quickly. Allow to stand twenty minutes or longer at room temperature. Attach the free rubber tube from "D" to a suction pump and draw air slowly through all tubes for one minute, to collect in the 0.02 N acid any free ammonia present in tubes "A" and "C." Shut off the suction and add to tubes "A" and "C," 10 cc. of sodium or potassium carbonate solution or about 5 gm. of one of those compounds
that air
second
minute.

In the absence of an air-flow meter, a suction pump capable of reducing pressure to 50 mm. Hg and working at capacity will suffice to effect a satisfactory air-flow.

When the aération is complete the acid remaining unneutralized in tubes "B" and "D" is titrated with 0.02 N alkali. The precaution must be taken to turn off the suction before disconnecting the tubes (center one first) to prevent back suction.

3. *Calculation*.—The number of cc. 0.02 N acid neutralized in tube "B," multiplied by 0.0056, gives the percentage of both ammonia and urea nitrogen; the acid neutralized in "D" tube, multiplied by 0.0056, gives

¹ An acetate buffer mixture which is much cleaner than caprylic alcohol may be used as follows:

Diluted acetic acid	60 cc.
Ammonium chloride	60 cc.

the per cent of ammonia nitrogen. The difference between the two ("B" minus "D") is the percentage of urea nitrogen; this figure multiplied by 2.144 gives the percentage of urea. To obtain the per cent of ammonia, multiply the figure for ammonia nitrogen by 1.216.

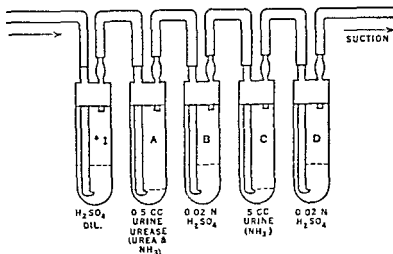


FIG. 13.

V. Ammonia Nitrogen.—A. Direct Colorimetric Method (Gentzkow assisted by Masen).—1. Reagents.—Those used in Method A for Urea Nitrogen, page 174.

2. Procedure.—To one volume of urine add 8 volumes of water, 1 gm. of Norit (special powdered charcoal), one-half volume of 0.66 N sulfuric acid and one-half volume of sodium tungstate solution, mixing after each addition. Filter, returning the first filtrate to the filter to insure absolute clarity. Transfer 1 cc. of the filtrate to a test tube graduated at 20 and 25 cc. In another similar tube place 5 cc. of the working ammonium sulfate
mark.
25-cc.

stand fifteen minutes, then compare in the usual manner, using the Wratten No. 75 filter with the visual colorimeter.

3. Calculation.— $\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 75 = \text{mg. ammonia nitrogen per 100 cc. of urine.}$ This figure multiplied by 1.216 gives the mg. ammonia per 100 cc. of urine.

B. Colorimetric Method Using Permutit (Gentzkow-Masen).—1. Reagents.—Same as in Method A and in addition:

- Permutit*
- Sodium hydroxide, 10 per cent.*

2. Procedure.—Place 2 cc. of urine, 2 gm. of permutit and 5 cc. of water in a 100-cc. volumetric flask. Shake gently for five minutes to absorb the ammonia. Add 20 to 30 cc. of water, mix and decant, avoiding any loss of permutit. Again wash with the same amount of water and decant as completely as possible. Next add 2 cc. of 10 per cent sodium hydroxide and 5 cc. of water. Mix, let stand 10 minutes, dilute to the mark and mix. Using 5 cc. of this dilution, proceed as in Method A.

3. Calculations.—The same as in Method A.

C. Aëration Method.—Ammonia nitrogen may be determined at the same time as Urea Nitrogen in B, page 175. Multiply the number of cc. of 0.02 N acid neutralized in tube "D" by the factor 0.0056 to obtain the percentage of ammonia nitrogen. The average content of ammonia nitrogen in normal urine is about 0.85 gm. per twenty-four hours; this is 4 to 5 per cent of the total urinary nitrogen.

VI. Uric Acid.—Direct Colorimetric Method (Folin).—1. Reagents.—(a) *Uric Acid Reagent*.—Place 100 gm. of sodium tungstate (C.P. molybdate-free) in a 500-cc. Florence flask. Mix 32 to 33 cc. of 85 per cent phosphoric acid with 150 cc. of water and add to the tungstate. Boil gently with a reflux condenser for fifty to sixty minutes. A flask and condenser with ground glass joint is best. If not available use a well washed cork-stopper for the connection. Next add 2 to 3 drops of bromine to decolorize the reagent, boil off the excess bromine, cool, and then dilute to 500 cc. Test the reagent as follows: To 5 cc. of water add 4 cc. of the reagent and 10 cc. of the urea-cyanide solution. Let stand fifteen minutes. No blue color should form. If it does, add 5 gm. of sodium tungstate to the remaining reagent, boil again for ten to fifteen minutes, decolorize with bromine if necessary, cool and dilute to volume as before.

(b) *Urea-cyanide Solution*.—To 75 gm. of sodium cyanide (C.P.) in a 2000-cc. beaker add 700 cc. of water and stir until solution is complete. Add 300 gm. of urea and stir. Next add 4 to 5 gm. of calcium oxide and stir for ten minutes. Let stand overnight, then filter. Add to the filtrate 2 gm. of lithium oxalate, shake occasionally for ten to fifteen minutes, then filter. Keep in the refrigerator.

This solution is very poisonous. It should be so marked. Never pipet such solutions. In discarding, pour cyanide solutions directly into the drain of the sink and flush at once with cold water.

(c) *Standard Uric Acid Solution*.—A stock solution is first prepared. Transfer exactly 1 gm. of uric acid by means of a small funnel to a liter volumetric flask. Dissolve 0.6 gm. lithium carbonate in 150 cc. of water in a 250-cc. flask. After solution is complete, heat to 60° C. Pour the lithium carbonate solution on to the uric acid, washing into the flask that remaining in the funnel. Shake until the uric acid is dissolved, warming under running hot water if necessary. Solution should be complete in five minutes. Cool under running water without delay. Add 20 cc. of 10 per cent formaldehyde solution and enough water to half fill the flask. Add a few drops of methyl orange indicator solution and lastly from a pipet slowly and with constant shaking, 25 cc. of 1 N sulfuric acid. The indicator should change to pink while 2 to 3 cc. of acid remain in the pipet, showing that the excess of acid is not too great. Dilute to volume, mix thoroughly, and transfer to a brown, glass-stoppered bottle. In the refrigerator and protected from light this stock solution keeps indefinitely.

(d) *Working Standard*.—Dilute 1 cc. of stock solution to 250 cc. with water. One cc. contains 0.004 mg. uric acid. This solution will keep for one month in the refrigerator.

2. Procedure.—Dilute 1 cc. of urine to 100 cc. in a volumetric flask. Using test tubes graduated at 25 cc. place 5 cc. of urine in one, 3 cc. of urine plus 2 cc. of water in another, and 5 cc. of the working uric acid standard in a third. To each add 10 cc. of the urea-cyanide solution, mix,

and then 4 cc. of the uric acid reagent. Let stand fifteen to twenty-five minutes, dilute to volume, and compare in the usual manner.

With the photoelectric colorimeter a filter with maximum transmission at 520 $m\mu$ should be used.

3. Calculation.—
$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 40, \text{ if 5 cc. of diluted urine were used (or } \times 66\frac{2}{3} \text{ if 3 cc. were used)} = \text{mg. uric acid per 100 cc. of urine.}$$

VII. Creatinine (Folin).—On adding picric acid and sodium hydroxide to a solution containing creatinine a deep red color is produced. The intensity of this color produced in a specimen of urine is compared with that in a standard creatinine solution. No substances in normal or pathological human urines interfere so far as is known.

1. Reagents.—The same as those used for creatinine in blood, page 205.

2. Procedure.—With an Ostwald pipet transfer 1 cc. of the stock standard creatinine solution (1 mg. per cc.) to a 100-cc. volumetric flask. In another similar flask place 1 cc. of urine. To each add 1.5 cc. of the 10 per cent sodium hydroxide and 20 cc. of 1 per cent picric acid solution. Shake, let stand ten to fifteen minutes, dilute to volume, mix and compare in the colorimeter in the usual manner.

For visual colorimeters a filter made of Wratten gelatin filters Nos. 45H and 58-B2 is of great help in making the comparison. The maximum transmission of this combination filter is at 520 $m\mu$. With photoelectric colorimeters a 520 $m\mu$ filter must be used.

3. Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 100 = \text{mg creatinine per 100 cc. of urine}$$

VIII. Creatine (Folin).—Normal adults, unless on a heavy diet of animal protein, excrete only traces of creatine in the urine. Children and patients with fever may excrete considerable quantities. Creatine is determined by first converting it into its anhydride, creatinine, by heating under pressure with dilute mineral or other acids. At a temperature of 117° to 120° C., equivalent to heating under a pressure of 1 kg. per square centimeter (14 pounds per square inch) conversion is complete in fifteen minutes.

1. Reagents.—Those used in creatinine determination, page 205.

2. Procedure.—Measure 1 cc. of urine into a 100-cc. volumetric flask and add 20 cc. of the picric acid solution. Cover the mouth of the flask with tinfoil and heat in the autoclave at 115° to 120° C. for twenty minutes. Cool, add 1.5 cc. of sodium hydroxide solution and continue as with creatinine.

3. Calculation.—Calculate the total creatinine (creatinine originally present plus that converted from creatine) as in the preceding determination. The difference between this reading and that for creatinine multiplied by 1.16 gives the amount of creatine.

IX. Glucose.—A. Benedict Method.—1. Reagents.—(a) Sodium carbonate, crystals or monohydrated salt.

(b) *Fumice* or *talc*.

(c) *Benedict's reagent, quantitative*.

Copper sulfate	18 gm.
Sodium carbonate (74 gm. line salt)	87 gm.
Sodium citrate	200 gm.
Potassium thiocyanate	125 gm.
Potassium ferrocyanide, 5 per cent solution	5 cc.
Distilled water to make	1000 cc.

With the aid of heat, dissolve the carbonate, citrate, and thiocyanate in enough water to make about 800 cc. of solution. Filter if necessary. Dissolve the copper sulfate separately in about 100 cc. of water, and pour this solution slowly into the first one, with constant stirring. Add the ferrocyanide solution, cool to room temperature, and make up to 1000 cc. in a volumetric flask. Of the various salts only the copper sulfate must be weighed with extreme accuracy. Exactly 25 cc. of this reagent are reduced by 50 mg. of glucose.

2. **Procedure.**—Dilute 10 cc. of clear urine to 100 cc. with water, unless the sugar content is known to be low. Fill a 50-cc. buret with this diluted urine. Measure exactly 25 cc. of the Benedict's reagent into a porcelain evaporating dish, add about 15 gm. of crystalline sodium carbonate (half that amount of the monohydrated salt, or 6 gm. of the anhydrous), and a small amount of the monohydrated salt, or 6 gm. of the anhydrous), and keep the mixture boiling vigorously during the entire titration. As soon as the carbonate is completely dissolved add the diluted urine from the buret at the rate of 1 cc. every ten seconds at first, until a chalk-white solution is formed. It is then

ars from the solution. Half-minute intervals must be allowed to elapse between additions of urine in the final steps of the titration. Water may be added if the mixture becomes too concentrated.* Determine the end point while the solution is still hot; upon cooling the solution tends to regain a bluish-green tint. With urine, the color at the end tends to be slightly yellowish, or yellowish-green due to urinary pigments.

Caution.—If chloroform has been used as a preservative, remove it by boiling a sample of urine for a few minutes and then diluting to its original volume.

3. **Calculation.**—When the urine is diluted 1 to 10, the following formula applies: $\frac{0.050}{N} \times 1000 = \text{per cent of glucose in original sample}$ where N is the number of cubic centimeters of diluted urine required to reduce 25 cc. of the reagent.

In general, $\frac{0.050}{X} \times 100 = \text{per cent of glucose}$, where X = number of cubic centimeters of undiluted urine required for the reduction.

B. Colorimetric Method—Modified from Folin and Svedberg.—1.

Reagents.—(a) Oxalic acid, 0.05 N.

(b) Lloyd's alkaloidal reagent.

(c) Permutil.

(d) Sodium hydroxide, 0.1 N.

* Additional sodium carbonate must be added if more than 25 cc. of diluted urine are used. The carbonate concentration must be kept at 25 per cent or above.

(e) *Alkaline tartrate solution*: Place 23.3 gm. of anhydrous sodium carbonate in a liter volumetric flask, add 200 cc. of distilled water, shake to dissolve. Add 8.7 gm. C.P. sodium tartrate and 7.34 gm. of sodium bicarbonate (NaHCO_3). Add distilled water to about 800 cc. Shake to dissolve. Make up to volume.

(f) *Copper solution*: Dissolve 16.7 gm. of crystalline CuSO_4 in a little distilled water. Make up to 500 cc. in volumetric flask.

(g) *Molybdate-phosphate solution*: See page 207.

(h) *Standard sugar solution*: Use the stronger working standard containing 0.4 mg. dextrose in 2 cc. (page 207).

2. *Procedure*.—To 5 cc. of urine in a 50-cc. Erlenmeyer flask add 5 cc. of 0.05 normal oxalic acid, 10 cc. reagent, and 2 gm. of permittit.

through a filter paper of fine pores. If the filtrate is not neutral, it is safest to make a preliminary titration of an aliquot with 0.1 N NaOH using phenolphthalein as the indicator. Place 2 cc. of the urine filtrate in a Folin sugar tube and add the requisite amount of 0.1 N sodium hydroxide, if any is called for by the preliminary titration. Transfer 2 cc. of the standard sugar solution to a second tube. To each add 2 cc. of alkaline tartrate-copper solution made by mixing 9 volumes of the tartrate with one volume of copper solution. This solution must be mixed freshly each day. Heat in boiling water for six minutes. Cool in an ice bath for three minutes. Immediately after the three minute cooling period add 2 cc. of molybdate-phosphate reagent. Dilute to volume with a dilute solution of the molybdate-phosphate reagent (stock solution diluted 1 to 5 with distilled water). Mix by inverting the tubes. Let stand ten minutes for color development. Compare in the colorimeter with the standard sugar solution.

3. Calculations.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 80 = \text{mg sugar per 100 cc. urine}$$

In the case of diabetic urines an aliquot of the filtrate from the Lloyd's reagent-permittit treatment is diluted with distilled water to such a volume that 2 cc. will contain from 0.2 to 0.8 mg. of dextrose.

X. *Total Acetone Bodies (Van Slyke)*.—This method combines Shaffer's oxidation of beta-hydroxybutyric acid to acetone with Denigé's precipitation of the latter as a basic mercuric sulfate compound.

1. *Reagents*.—(a) *Mercuric Sulfate*, 10 per cent solution: Dissolve 37.5 gm. of pure red mercuric oxide in 500 cc. of 4 N sulfuric acid.

(b) *Sulfuric Acid*, 50 volumes per cent: Dilute 250 cc. of H_2SO_4 , specific gravity 1.84, to 500 cc. with water. The concentration must be readjusted if necessary to make it 17 N by titration.

(c) *Calcium Hydroxide*, 10 per cent: Dissolve 50 gm. of fine, light Ca(OH)_2 in 500 cc. of water.

(d) *Potassium Dichromate*, 5 per cent: Dissolve 25 gm. of $\text{K}_2\text{Cr}_2\text{O}_7$ in 500 cc. of water.

2. *Procedure*.—Interfering substances must be removed and this is accomplished as follows: To 25 cc. of urine in a 250-cc. flask add 100 cc. of water, 50 cc. of 20 per cent copper sulfate solution and mix. Then add 50 cc. of 10 per cent calcium hydroxide suspension, shake, and test for

alkalinity. If not alkaline to litmus add more calcium hydroxide. Dilute to 250 cc. and allow to stand thirty minutes in order that the glucose may precipitate. Filter. This procedure will remove up to 8 per cent glucose. Urine containing more should be diluted enough to bring the glucose down to that figure. The copper sulfate removes substances other than glucose and should never be omitted even though glucose is absent. Test the filtrate for the presence of glucose by boiling a portion. A yellow cuprous oxide will be obtained if the glucose has not been completely removed.

Place in a 500-cc. pyrex Erlenmeyer flask 25 cc. of the urine filtrate. Add 100 cc. of water, 35 cc. of mercuric sulfate solution and 10 cc. of the 50 per cent sulfuric acid. Connect the flask with a reflux condenser having a straight condensing tube of 8 to 10 mm. diameter and heat to boiling. After boiling has begun, add 5 cc. of the 5 per cent dichromate solution *through the condenser tube*. Continue the boiling gently for one and a half hours. The yellow precipitate which forms consists of the mercury sulfate-chromate compound of acetone. It is collected on a Gooch crucible, washed with 200 cc. of cold water and dried for one hour at 110° C. The crucible is allowed to cool in room air and weighed.

3. Calculation.—When 25 cc. of urine filtrate, equivalent to 2.5 cc. of urine are used, multiply the weight in grams of precipitate by 24.8 to calculate the grams of acetone per liter of urine.

Test of Reagents.—When the complete total acetone bodies determination, including the preliminary copper sulfate treatment, is performed on a sample of distilled water instead of urine, no precipitate whatever should be obtained. This blank test must not be omitted.

XI. Chlorides (Modified Volhard-Harvey Titration).—1. Reagents.—(a) *Standard Silver Nitrate Solution*: Dissolve 29.06 gm. of silver nitrate in about 100 cc. of water in a liter volumetric flask. Add 250 cc. of concentrated nitric acid and 250 cc. of a saturated aqueous solution of ferric ammonium sulfate. Dilute to a volume of 1 liter. One cc. of this solution is equivalent to 0.01 gm. of sodium chloride.

(b) *Standard Ammonium Thiocyanate Solution*: Dissolve about 6.5 gm. of ammonium thiocyanate (NH_4SCN) in 800 cc. of distilled water. Titrate this solution against the standard silver nitrate solution. Then calculate the amount of water which must be added to the stock thiocyanate solution to make it one-half the strength of the silver nitrate solution. One cc. of the silver nitrate should equal 2 cc. of the ammonium thiocyanate; thus 1 cc. of the ammonium thiocyanate is equivalent to 0.005 gm. of sodium chloride.

2. Procedure.—To 5 cc. of urine, in a 250-cc. Erlenmeyer flask, add 100 cc. of water and 10 cc. of the silver nitrate solution. If the solution assumes a pinkish color, as it sometimes will, this may be dispelled by the addition of a few drops of saturated solution of potassium permanganate. Titrate with the thiocyanate until the appearance of the first salmon pink or brownish tint that persists several seconds. The most common error, producing low results, is failure to recognize the *temporary nature of the end point*. The first color that permeates the solution, for a few seconds after one shaking, is the true end point. In ketosis urine, enough acetoacetic acid may be present to give a red color with ferric iron; if so, acidify and boil for a few minutes.

3. Calculation.—

$2 A - B = \text{gm. of chloride calculated as NaCl per liter of urine.}$

$0.607 (2 A - B) = \text{gm. of Cl per liter of urine.}$

$17.1 (2 A - B) = \text{milli-equivalents of Cl per liter of urine.}$

$A = \text{cc. of silver nitrate added to the urine.}$

$B = \text{cc. of thiocyanate used in the titration.}$

XII. Inorganic Phosphate (Fiske and Subbarow).—1. Reagents.—The standard phosphate solution and the 0.25 per cent amino-naphtholsulfonic acid solution used for phosphorus in blood (page 215). The molybdic acid solution differs.

(a) *Molybdate Solution.*—Dissolve 25 gm. of ammonium molybdate in 200 cc. of water. Rinse into a liter volumetric flask containing 500 cc. of 5 N sulfuric acid. Dilute to the mark and mix.

2. *Procedure.*—Pipet 1 cc. of urine into a 100-cc. volumetric flask and 5 cc. of the standard phosphate solution (0.08 mg. P per cc.) into another. Dilute each to about 70 cc., then add 10 cc. of the molybdate solution and mix. Add 4 cc. of the amino-naphtholsulfonic acid solution, mix thoroughly and dilute to volume. Let stand five minutes, then compare in the colorimeter.

3. Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 40 = \text{mg. P per 100 cc. of urine}$$

XIII. Hydrogen Ion Concentration or pH.—The simplest method satisfactory for all clinical purposes is that using nitrazine paper (phenaphthazaine), procurable from E. R. Squibb and Sons.

With a clean glass rod transfer a drop of freshly-voided urine to the paper and spread evenly by stroking, or leave a small drop. The paper may be dipped into the urine three times and the excess shaken off.

After one minute compare with the color chart furnished which reads from pH 4.5 to 7.5 in 0.5 pH divisions. It is possible to interpolate to 0.25 pH without difficulty.

XIV. Ethyl Alcohol.—(See page 342.)

XV. Sulfonamides.—(See page 220.)

XVI. Analysis of Renal and Urinary Calculi.—If sufficiently large, the calculus should be cut in half to reveal any core or nucleus requiring analysis separately from the layers surrounding it.

A. Physical Examination.—Uric acid calculi are usually small, smooth, round lusterless pebbles. Upon crushing, the stones are always definitely yellow. Phosphate and carbonate stones are compact balls or large friable clay- or chalk-like masses. Oxalate stones vary considerably in form, but usually have a crystalline glint or smooth luster. Some are irregular, light-colored, and crystalline with sharp points. Others are externally dark-brown, with a grape seed or berry-like form. Still other forms are occasionally found. Cystine stones are usually white or pale yellow granules.

B. Chemical Examination.—1. *Reagents.*—In addition to concentrated hydrochloric, nitric, and sulfuric acids, and ammonia water, 23 per cent, the following reagents are required:

(a) *Sodium Acetate*, saturated aqueous solution.

(b) *Ammonium Oxalate*, 4 per cent.

(c) *Resorcinol*.

- (d) *Sodium Hydroxide*, 20 per cent.
- (e) *Sodium Carbonate*, 20 per cent.
- (f) *Sodium Cyanide*, 5 per cent.
- (g) *Sodium Nitroprusside*, a freshly prepared saturated solution.
- (h) *Nessler's Reagent*, see page 201.
- (i) *Uric Acid Reagent*, Folin's, see page 177.

(j) *Molybdate Reagent*. Dissolve 3.5 gm. of ammonium molybdate in 75 cc. of water and pour this solution into 25 cc. of concentrated nitric acid.

(k) *Magnesium Reagent*. Dissolve 5 mg. of p-nitrobenzene-azoresorcinol in 100 cc. of 1 N sodium hydroxide.

2. *Procedure*.—(a) *Carbonates*.—Pulverize the stone in a small, heavy pyrex test tube, with a glass rod. To part of the pulverized stone in a small test tube, add a few drops of concentrated hydrochloric acid. Carbonates will give an effervescence. Add an equal volume of distilled water. Warm gently to dissolve the stone. Centrifugalize to throw down any precipitate and divide the supernatant liquid into four portions. The solution may also be separated from the precipitate by drawing it through a capillary pipet plugged with a little cotton. In the analysis of calculi it is convenient to use test tubes of very small size. Small fermentation tubes 6 by 40 mm. are very useful in the manipulation of the small quantities of liquids involved.

(b) *Calcium Oxalate and Calcium*.—Add saturated solution of sodium acetate dropwise to one of the four portions. If calcium oxalate is present, a white precipitate or cloud forms. Add sodium acetate until no further precipitate appears. Centrifugalize, and to the supernatant, add another drop of sodium acetate solution. If no precipitate appears, add a few drops of the ammonium oxalate solution. A white precipitate indicates the presence of calcium in combination with an anion other than oxalate.

(c) *Phosphates*.—To another portion of the hydrochloric acid solution of the stone add ammonia until alkaline. Acidify with concentrated nitric acid, then add an equal volume of molybdate reagent. Warm to 60° C. A yellow precipitate shows the presence of phosphates.

(d) *Magnesium*.—To a third portion, add 3 drops of magnesium reagent, then make strongly alkaline with 20 per cent sodium hydroxide solution. A blue color or blue precipitate indicates magnesium.

(e) *Ammonia*.—Make a fourth portion alkaline with 20 per cent sodium hydroxide. Add a few drops of Nessler's reagent. Ammonia will give an orange brown precipitate. To confirm ammonia, add a few drops of 20 per cent sodium hydroxide to some of the powdered calculus in a small tube, then cover the tube with moist red litmus paper. Warm gently. Ammonia will turn the red litmus paper blue.

(f) *Uric Acid and Urates*.—To some of the powdered stone on a spot plate, or to an aqueous solution of the stone, add a few drops of 20 per cent sodium carbonate solution and a few drops of Folin's uric acid reagent. An immediate deep blue color indicates uric acid or urates, a pale blue color is negative. For a confirmatory test, apply the murexide reaction. Treat the powdered stone in a porcelain evaporating dish with several drops of concentrated nitric acid, evaporate to dryness on a water bath,

and add 2 to 3 drops of concentrated ammonia. Uric acid gives a deep yellow or orange-red residue with the nitric acid, turning purple with the ammonia. Xanthine gives a greenish-yellow residue with the nitric acid, turning to orange with ammonia.

(g) *Cystine*.—Cystine stones are rare. Ashed in a crucible, such stones give a piercing odor with the first puff of smoke. Another test is to add a drop of concentrated ammonia and a drop of 5 per cent sodium cyanide solution to some pulverized stone. After five minutes add 2 to 3 drops of sodium nitroprusside solution. A beet-red color indicates cystine.

(h) *Sulfonamides*.—Sulfonamides in stones may be detected by dissolving the stone in dilute hydrochloric acid and applying the tests for the sulfonamides as given on page 220.

CHAPTER XVI

QUANTITATIVE CHEMICAL EXAMINATION OF THE BLOOD

By CLEON J. GENTZKOW and JOHN M. MASEN

INTRODUCTION

MANY methods have been proposed for the determination of the various chemical constituents of the blood, differing greatly in complexity of materials and apparatus and in the degree of skill required for their performance. As one would suppose, they also differ widely in the degree of accuracy obtained. Those of greatest specificity and accuracy are often the most difficult and elaborate, suitable for research rather than clinical purposes. On the other hand, the over-simplification often attempted may result in the introduction of gross error.

The procedures presented in this Chapter have been selected from the almost endless variety available because: (1) The accuracy obtained is at least sufficient for all clinical purposes; (2) Results are reproducible; (3) They can be performed by technicians with a minimum of supervision, and (4) The reagents and apparatus required are available in most clinical laboratories or are easily obtainable.

COLORIMETRY

Most methods for determining the chemical constituents of the blood are based upon colorimetry, in which the color produced by the unknown substance is compared with that produced by a known amount of the same substance, assuming in each instance that the intensity of color is proportional to the amount of the color-producing substance present. Two general methods are used for measuring this color intensity:

1. The visual method, in which the colors are matched by the eye using a colorimeter of the Duboseq type.

2. The photoelectric method, in which the intensity of the color is measured by determining the amount of electrical current developed by a photoelectric cell when the light transmitted by the colored solution falls upon it.

All of the earlier methods are based upon visual colorimetry using a Duboseq type colorimeter and this particular method of color measurement is still used in the majority of laboratories. However, in recent years the photoelectric colorimeter has reached a high degree of perfection, and many of the newer methods are based upon the use of this instrument. Actually, the accuracy resulting from the use of a photoelectric colorimeter is no greater than that obtained using a visual colorimeter. It has been assumed, erroneously however, that the principal source of error in colorimetry is the inability of the eye to match colors accurately, and that this can be eliminated by substituting the photoelectric cell for the eye. As a matter of fact the principal source of error results from the fact that color development is not always strictly proportional to the concentration of

color producing substance present. In most instances this error is far greater than that due to the inability of the eye to match colors accurately. Furthermore, while it is conceded that in color matching the eye may be an imperfect instrument, the photoelectric cell as a substitute may be equally imperfect. The lack of proportionality in the response of the photoelectric cell to changing light intensities which result from variations in the color concentration is one source of error. Additional sources of error in the cheaper instruments result from inaccurately calibrated galvanometers, stray thermoelectric currents, and heating and fatigue effects in the photocell.

The photoelectric colorimeter has, however, great convenience and speed of operation. The cells or cuvetts in which the color of the final solution is measured may be simply matched test tubes in which all of the reactions leading to the final color development are carried out. To read the color, it is then only necessary to insert these tubes in the instrument, instead of transferring the solution to specially prepared cups or cuvetts as is required in visual colorimetry. Also very faint colors can be measured more accurately than by the eye, and in certain cases, where an interfering turbidity exists, this may be eliminated by calibrating the instrument with a proper blank. When more than one color is present, through the use of the proper color filters, the amount of each in the presence of the other may be determined. For example, in a specimen of blood containing methemoglobin in the presence of oxyhemoglobin, the relative amounts of each may be measured in this manner. Where a substance has a specific absorption band, the selection of the proper color filter in conjunction with the photoelectric colorimeter, will frequently render the measurement of the color more specific than is the case in visual colorimetry using white light.

I. Visual Colorimetry.—Most colorimeters in common use are of the Duboscq type. There are numerous modifications of this instrument, all of them more convenient and accurate than the original. The principle of operation, however, in all of these modifications is the same as that of the original. The solutions to be compared are placed in glass cups which can be raised or lowered by means of a rack and pinion movement. The method of operation may be inferred from a study of Figures 14 and 15. As the cups are raised or lowered the ends of clear glass plungers are immersed to varying depths in the fluid, thus varying the thickness of the layer of fluid between the ends of the plungers and the bottoms of the cups. The thickness of this layer is indicated on a scale, attached to the rack and pinion movement, which moves in unison with the cups. Light is reflected by means of a mirror beneath the cups through the solution and the long axis of the plungers into a series of prisms. The prisms bring the light from the two cups into a single field viewed by an ocular. The field appears as a circle divided in half by a fine line (Fig 15). One-half of the field is illuminated by the light passing through one of the cups, and the other half of the field is illuminated by the light from the other cup. When the depth of fluid in the two cups is the same and the color concentration of each fluid is also the same, the color of the two halves of the field will be equal. However, if one color is stronger than the other, the field illuminated by light passing through this solution will be darker than the other, and the two fields may be equalized by adjusting the depth of the solution by

moving one of the cups. The scales will then read the respective heights of the two colored solutions, and the concentrations of the colors will be inversely proportional to the scale readings. The light reflected by the

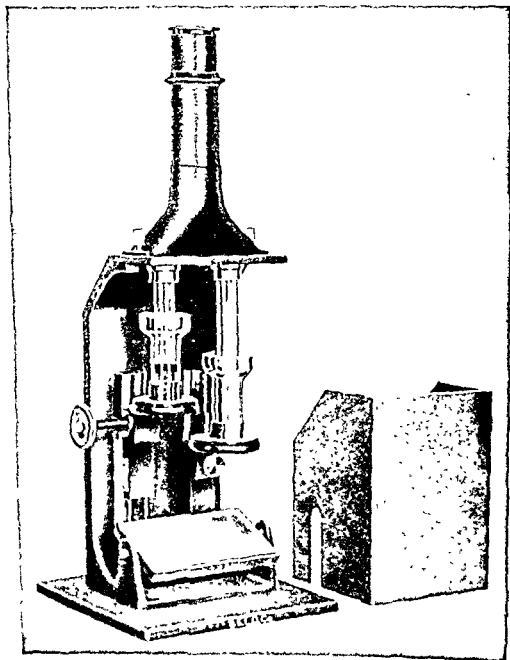


FIG. 14. Duboscq colorimeter. (Courtesy of Bausch & Lomb.)

1. **Standardization.** Because of the variation in colorimetric readings made under different conditions, the colorimeter should be standardized before use, and by the person who is to use it. The fact that a colorimeter has been standardized in the morning is no evidence that it will remain so,

If, now, one solution is of known concentration and is used as a standard

$$c_u = \frac{c_s \times R_s}{R_u} \quad (4)$$

Where c_u = concentration of unknown,
 c_s = concentration of standard,
 R_u = reading (depth) of unknown, and
 R_s = reading (depth) of standard.

Example.—A standard solution of sugar containing 0.2 mg. when treated in such a manner as to develop a color is placed in one cup of the colorimeter and set to read 20 on the scale of the colorimeter. The color of an unknown amount of sugar in a sample of blood is developed in the same manner as the standard. This unknown solution is then matched against the standard and the scale reading of the unknown is found to be 10. By substitution:

$$c_u = \frac{0.2 \times 20}{10} = 0.4 \text{ mg. sugar}$$

Note the inverse relationship between reading and concentration, a reading of 10 for the unknown which is one-half the reading of the standard gives a concentration of 0.4, exactly twice that of the standard.

In most work in blood chemistry results are expressed in milligrams per 100 cc. of whole blood or serum. Equation (4) must be modified to allow for this and so becomes

$$c_u = \frac{c_s \times R_s}{R_u} \times \frac{100}{V} \quad (5)$$

where V is the volume of the sample of whole blood or serum used. This formula holds if both standard and unknown are diluted to the same final volume. In some determinations, that of creatinine for example, this is not the case. A dilution factor must be introduced and the formula becomes

$$c_u = \frac{c_s \times R_s}{R_u} \times \frac{100}{V} \times \frac{D_u}{D_s} \quad (6)$$

where D_u and D_s are the final volumes of unknown and standard.

This is the final and complete equation upon which all colorimetric calculations are based.

Examples.—Carrying on further with the example given above the actual values are:

$c_s = 0.2$ mg sugar	$V = 0.2$ (2 cc of filtrate = 0.2 cc blood)
$R_s = 20$	$D_u = 25$
$R_u = 10$	$D_s = 25$

Substituting in the formula

$$c_u \text{ (mg / 100 cc.)} = \frac{0.2 \times 20}{10} \times \frac{100}{0.2} \times \frac{25}{25} = 200$$

As an example in which the volumes of the final colored solutions differ, let us take the determination of creatinine with the following values:

$c_s = 0.03$ mg	$V = 1$ (10 cc. of filtrate = 1 cc. blood)
$R_s = 20$	$D_u = 15$
$R_u = 10$	$D_s = 30$

Substituting:

$$c_u \text{ (mg / 100 cc.)} = \frac{0.03 \times 20}{10} \times \frac{100}{1} \times \frac{15}{30} = 3$$

In actual practice all of the above factors are combined into a single one and the formula given with each determination becomes a simple one. For sugar it becomes

$$\frac{R_s}{R_u} \times 100 = \text{mg. sugar per 100 cc. of whole blood}$$

when the standard contains 0.2 mg. of glucose.

That for creatinine becomes

$$\frac{R_s}{R_u} \times 15 = \text{mg creatinine per 100 cc. of whole blood}$$

using the standard containing 0.03 mg. of creatinine.

Certain principles should always be held in mind. One of these is the fact that while Beer's law is exact within reasonable limits, it is not strictly true when two solutions of widely differing concentrations are compared. These deviations from Beer's law are due to such effects as the association of molecules and the formation of complexes. For this reason, the ratio of the concentration of the solutions should not be greater than 2 to 1. If at all possible, the standard should be of such a strength that the ratio of the concentrations is not greater than 1.5 to 1. Lambert's law is rigidly true for all depths of liquid.

5. **Color Filters.**—Color filters may be used in the visual colorimeter to increase sensitivity and selectivity in the same manner as in photoelectric colorimetry; in fact many of the advantages of photoelectric colorimetry may be obtained for the visual method by the use of these filters. Their selection and use will be considered more fully in the section which follows.

II. **Photoelectric Colorimetry.**—1. **Principles.**—Photoelectric colorimetry is based upon the property possessed by photoelectric cells of generating a small electric current which is proportional to the amount of light falling on the cell. Since the current so generated is quite small, usually a fraction of a milliamper, it is necessary that a sensitive galvanometer be used for its measurement, and if a galvanometer is connected in series with the photoelectric cell, the reading of the galvanometer will be proportional to the amount of light falling on the cell. If a glass cuvet or tube containing a colored solution whose intensity it is desired to measure is interposed between the light source and the photocell in such a manner that the only light which can reach the photocell must pass through the colored solution, there will be a change in the reading of the galvanometer which will be proportional to the intensity of the color of the solution. The deeper the color, the more light will be absorbed, with a consequent reduction in the amount reaching the photocell and a corresponding decrease in the reading of the galvanometer.

2. **Types.**—Commercial photoelectric colorimeters are of two general types:

(a) *The Single Cell Colorimeter.*—This type of instrument consists of a single photoelectric cell connected in series with a galvanometer graduated in equal divisions from 0 to 100. An instrument of this kind is shown in Figure 16. The source of light consists of a tungsten filament incandescent light, the current being obtained either from a storage battery or from the regular 110 volt alternating current supply stabilized by means of a constant voltage regulator. This lamp may be connected in series with a variable rheostat by means of which the light striking the photocell may

be regulated, or the regulation may be by means of a variable shunt across the galvanometer terminals, or by an iris diaphragm between the light source and photocell.

(b) *Double Photocell Instrument.*—The second type of instrument is one in which two photoelectric cells are connected with the galvanometer in such a manner that the current flow of the one cell is opposed by that

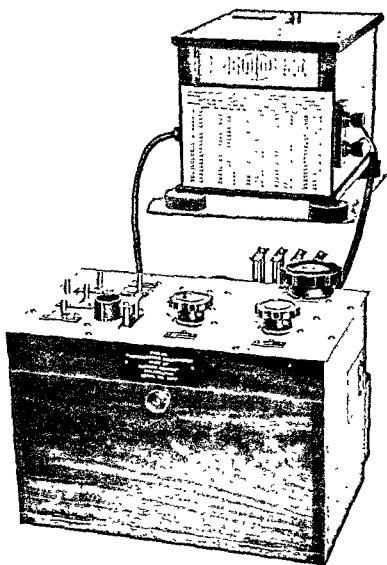


FIG. 16 —A single-cell photoelectric colorimeter with galvanometer (Evelyn). (Courtesy of The Rubicon Company, Philadelphia)

of the other.
galvanometer
of instrument.
calibrated resist

reading from 0 to 100. The light is placed in such a manner as to illuminate both cells equally and when the current is adjusted to equality with a

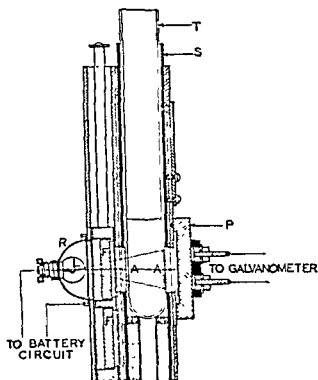


FIG. 17. - Cross-sectional diagram of the main structural unit of photoelectric colorimeter shown in Figure 16. A beam of light from the lamp *L* in reflector *R* passes through the glass color filter *F*, and then through the colored solution in the absorption test tube *T* before falling on photocell *P*. The current from the photocell is registered by a galvanometer (not shown). The test tube *T* fits into a lakelite sleeve *S* pierced by two rectangular apertures *A*, which define the cross-section of the light beam. (Courtesy of The Rubicon Company, Philadelphia)

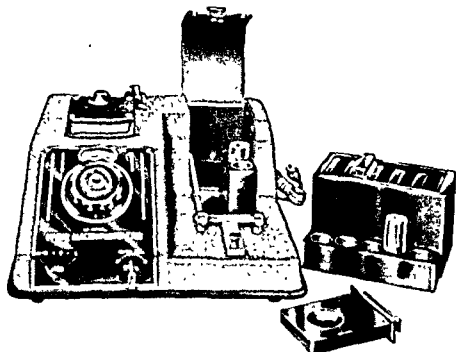


FIG. 16 - A photoelectric colorimeter with a battery and a potentiometer circuit. (Courtesy of The Rubicon Company, Philadelphia)

blank solution before one of the cells the galvanometer will read zero, indicating no current flow through the galvanometer. When, however, the blank solution is replaced by the test solution, the light reaching this cell will be reduced owing to absorption by the colored solution, the current generated will be less than that from the other cell, and the galvanometer will no longer read zero. Equality of current flow through the two cells is restored by adjusting the calibrated resistor and is indicated by return of the galvanometer to the zero position. The reading of the resistor will then be a function of the light transmitted through the test solution, which in turn is dependent upon the intensity of the color of this solution. Figure 18 illustrates a photoelectric colorimeter of the two-celled type.

3. *Respective Merits.*—The relative advantages and disadvantages of the two types of instruments are:

(a) In the case of the single cell colorimeter a relatively stable source of light is required as a change in the light source will cause a change in the reading of the galvanometer. This does not occur with the double-cell instrument for the change of light will be the same on each cell, and because of the type of electrical circuit used, this effect will be cancelled. It is therefore necessary with the single-cell instrument to use a small light source with a large capacity battery, or a high grade voltage regulator when the line voltage is used.

(b) An accurately calibrated galvanometer is required with the single-cell instrument, since the transmission value is obtained from the reading of the galvanometer, and the accuracy of this value will depend upon the degree of accuracy of the galvanometer calibration. Such a galvanometer is relatively difficult and expensive to manufacture. In the two-cell instrument, however, the results are unaffected by the accuracy of the galvanometer calibration, since the galvanometer is used merely to indicate the point of balance obtained by adjusting the calibrated resistor, the transmission values being obtained from the reading of this resistor. Therefore, the accuracy is dependent upon the calibration of the resistor which is cheaper and simpler to manufacture than the galvanometer.

(c) The single-cell instrument is preferable for following rapidly changing reactions in which maximum color development may occur within a few seconds following admixture of reagents, the maximum reading of the galvanometer being recorded. With the two-cell instrument it is more difficult to follow these rapid changes since it is necessary to obtain the reading by adjusting the galvanometer to zero by means of the variable resistor. Because of the time required for this adjustment, it is difficult to determine at which point maximum color develops when it changes so rapidly as it does, for instance, in the vitamin A determination by the Carr-Price reaction or in that for vitamin C by means of the dichloro-indophenol reaction. Also the samples may be read with greater speed in the one-cell instrument since fewer manipulations are involved in making the reading.

4. *Method of Using the Photoelectric Colorimeter.*—(a) *Single Cell Colorimeter.*—Turn on the instrument about five to ten minutes before making a reading in order that all parts of the electrical circuit may come to equilibrium. Make sure that the proper color filter has previously been inserted. Neglect of this precaution may result in irreparable damage to the galvanometer. Insert the tube containing the blank and adjust until

the galvanometer reads 100. The blank usually consists of all the reagents used in the test except that an equivalent amount of distilled water is substituted for the sample. Remove the blank, insert the test solution and record the reading. If a standard is used, repeat the procedure with it. After each reading check the instrument to see that the reading has not changed from the initial setting of 100.

(b) *The Double Cell Colorimeter.*—The instrument is turned on with the color filter in place in the same manner as the single cell instrument. Insert the blank and with the calibrated resistor set to 100 adjust the instrument until the galvanometer reads zero. Then remove the blank, insert the sample and adjust the calibrated resistor until the reading of the galvanometer is again restored to zero. The reading of the calibrated resistor at this point will be the transmission reading of the sample. Obtain the reading of the standard in the same manner as the unknown.

5. *Calculations.*—A standard solution may be used in the same manner as in visual colorimetry. The readings obtained represent transmission of light through the colored solution, the greater the density of the color, the smaller will be the amount of light striking the photocell with a corresponding decrease in the readings. The concentration of the color producing substance is not, however, proportional to this transmission value but is, instead, proportional to the density. Density may be obtained from the

formula of Lambert— D (density) = $\log \frac{I_0}{I} = \log I_0 - \log I$, in which I_0 is the incident light, represented in this instance by 100, the reading of galvanometer or resistor with the blank, and I , the transmission or reading of the test sample. Since the log of 100 is 2, then 2 minus the log of the reading of the sample equals the density which is directly proportional to the concentration of the color producing substance. Note that the proportion in this case will be direct rather than inverse as in visual colorimetry, and may be expressed mathematically as:

$$\frac{c_s}{c_u} = \frac{R_u}{R_s} \quad (1)$$

or

$$c_u = \frac{c_s \times R_u}{R_s} \quad (2) \text{ where}$$

R_s = 2 - log of the transmission reading of the standard

R_u = 2 - log of the transmission reading of the unknown

c_s = Concentration of the standard, and

c_u = Concentration of the unknown.

All of the other factors of equation (6) on page 190 are the same and the complete equation for photoelectric colorimetry may be written:

$$c_u = \frac{c_s \times R_u}{R_s} \times \frac{100}{V} \times \frac{D_s}{D_u} \quad (3)$$

It is convenient to construct a table giving the values of 2 - log of reading for all transmissions of the instrument.

A second method of obtaining the concentration of the unknown is to prepare a series of standards of varying concentrations to cover the maximum range usually found in the unknowns. As a general rule, five to ten standard solutions are prepared. If the reading for each is plotted graphically against its concentration a calibration curve is readily formed; the

reading of a solution of unknown concentration can then be referred to this curve to determine its concentration.

This method would appear to be much simpler than the use of a standard prepared for each series of determinations, since, once a calibration curve has been prepared, the use of a standard can be omitted. However, color production is influenced by many variables, such as time, temperature, the presence of other compounds, the nature, age and composition of the reagent solutions, and even the rate of addition of the reagents in some cases. A curve prepared one day under a certain set of conditions may not be valid the next day, week or month, since conditions may be different and uncontrollable. When, however, the results are obtained by comparison with a standard prepared simultaneously and under the same conditions as the unknown many of these variables are cancelled.

The calibration curve method should be used only with those colors that are extremely stable and reproducible, or where the preparation of a standard solution each time is difficult or expensive. The use of a calibration curve is also to be recommended in certain instances where the color produced is not proportional to the concentration of the color producing substance. The use of a calibration curve will correct any error due to this lack of proportionality.

6. Color Filters.—It is well known that colored substances owe their attribute of "color" to the fact that they absorb selectively certain parts of the visible spectrum. Thus a blue color is blue because it transmits the blue portion of the spectrum and absorbs the red portion. Hence a red

by the blue solution only;
 is absorbed light and result
 eliminating these unabsorbed
 wave lengths by means of the proper filter, the sensitivity of the method is correspondingly increased. In the case of a red solution, blue light is absorbed, and, therefore, by using a blue filter greater sensitivity is obtained. The above examples serve to illustrate only one particular factor, sensitivity, governing the selection of the proper filter. Certain solutions, for example, possess specific absorption bands in the visible spectrum and a filter transmitting this particular band will give the desired result. Hemoglobin is an example. This substance absorbs a very narrow wave band centered at 540 $m\mu$ in the green, and a filter transmitting this particular wave band will not only increase the sensitivity but the specificity as well. Another factor governing the selection of the proper filter is the possible presence of another contaminating color, either from the reagents, or as a result of a reaction between the reagents and some other substance present in the test solution. For example, in the determination of creatinine by the reaction with picric acid, creatinine picrate, a red-colored compound is formed. Therefore, from the standpoint of maximum sensitivity a blue filter should be used. However, a large excess of picric acid, which is yellow, is required for this reaction. This yellow picric acid also shows strong absorption in the blue portion of the spectrum and the use of a blue filter would cause a considerable error as one would be measuring the concentration of the picric acid as well as that of the creatinine picrate. At wave length 520 $m\mu$ in the green, while the absorption of creatinine picrate is not as great as it is with the shorter wave lengths in the blue, the absorption is nevertheless appreciable, while the absorption due to

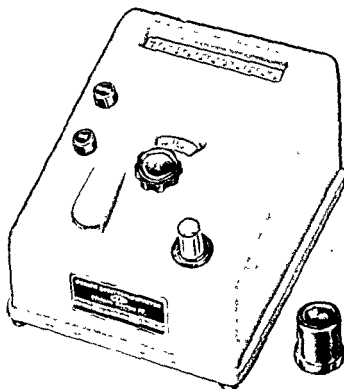


FIG. 10.—Junior clinical spectrophotometer, Model 6. (Courtesy of the Coleman Electric Co.)

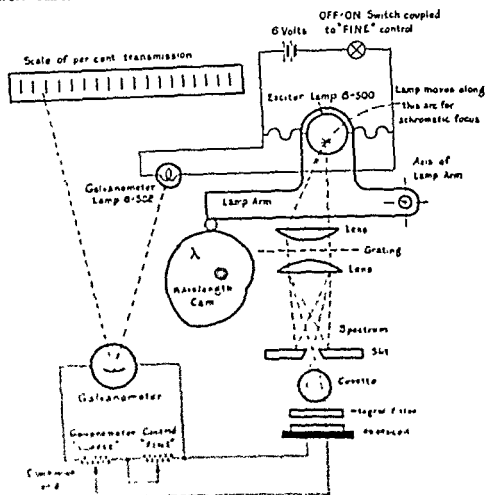


FIG. 11.—Schematic diagram of the Junior clinical spectrophotometer, Model 6. (Courtesy of the Coleman Electric Co.)

the picric acid at this wave length is negligible. Therefore, for this determination, a filter passing this wave band is preferable to the more sensitive filters in the blue.

These are but a few of the factors governing the selection of the proper filter. Final selection of that filter which gives greatest sensitivity and specificity in any particular procedure can only be determined by a spectrophotometric analysis of the solution under examination. In those photocolorimetric methods included in this book, the filters to be used are specified.

III. Spectrophotometers in Colorimetry.—The Medical Department has adopted as a standard instrument for issue to certain specified installations the Coleman Junior Clinical Spectrophotometer, Model 6. This instrument is rugged and practical, having all of the advantages of a photoelectric colorimeter in addition to many of its own.

Filters are not required since it uses a replica grating and fixed slit by means of which narrow bands of wave lengths may be isolated throughout the visible spectrum.

Figures 19 and 20 illustrate the instrument and a diagrammatic representation of the light path and functioning parts.

COLLECTION AND PRESERVATION OF SPECIMENS

I. Collection.—For most determinations the blood is obtained by venepuncture after the patient has fasted ten to twelve hours. For convenience the blood is usually drawn before breakfast.

When venepuncture is difficult or impossible resort may be had to micro methods using 0.1 cc. of blood secured by finger puncture.

II. Anticoagulants.—In using whole blood coagulation must be prevented. Lithium oxalate is the best anticoagulant for most purposes since it interferes least with subsequent procedures. For each cc. of blood, use 1.5 mg. of the salt. Potassium oxalate, 2 mg. per cc., may be used instead but is less satisfactory. Citrates should not be used.

A convenient method of adding the anticoagulant is to prepare collecting tubes in advance. Using test tubes of large diameter, small flasks, bottles or short, heavy, flat-bottomed sputum bottles (Item 40830, Medical Department Supply Catalog) introduce 1 cc. of 1.5 per cent lithium oxalate solution (2 per cent potassium oxalate) into the tube. This amount is sufficient for 5 to 12 cc. of blood and does not produce too great an excess. Dry in an incubator or oven overnight. Do not permit the temperature to exceed 55° C. since at that point and above some of the oxalate may be converted to carbonate. By this procedure the oxalate is distributed in a thin layer and in a finely divided state over the lower portion of the tube, going into solution as soon as the blood touches it.

Discharge the blood into the oxalate tube as soon as it is drawn, and mix most thoroughly. Even a slight delay may result in some clot formation.

III. Preservatives.—Specimens should be analyzed as promptly as possible before hydrolytic changes occur. If immediate analysis is impos-

a few drops of toluene or xylene and place it in the refrigerator. So prepared a filtrate keeps well for twenty-four hours.

If specimens are to be sent to a distant laboratory, sodium fluoride in the proportion of 10 mg. per cc. of blood may be used as a preservative. The blood must be collected and handled with strict asepsis for fluoride will not prevent decomposition due to bacterial contamination. Since fluoride is an anticoagulant as well as a preservative, no other need be used.

Keidel tubes containing the proper amount of fluoride are available from laboratory supply companies and are very convenient for collecting and shipping specimens of whole blood.

The preservative effect of fluoride is due to its inhibitory effect on enzymes. For this reason, blood so preserved cannot be used for urea determinations since fluoride inhibits the action of urease.

IV. Serum Specimens.—When serum specimens are required, no anticoagulant is used. Draw blood by venepuncture using a *dry* syringe and needle, and transfer it immediately to a chemically-clean, dry test tube or centrifuge tube. Do not use so much pressure on the syringe piston that a froth is formed. Allow the tube to stand for a short time until the clot forms, then chill in ice water and place in the refrigerator. When the clot has contracted, gently loosen it with a glass rod or applicator, if necessary, and centrifugalize. Pipet or pour off the clear serum into a dry, clean tube.

If no centrifuge is available, place the tube containing the blood nearly horizontal so that a long, slanting surface is produced. Do not move it until clotting is complete, then chill and place in the refrigerator overnight. Pour off the clear serum from the side of the tube opposite the slant. Syringes, needles and tubes *must* be clean and dry, otherwise hemolysis will occur vitiating many of the results of subsequent analyses.

CHEMICAL EXAMINATIONS

When the non-protein constituents of blood are to be determined the protein is removed by means of a precipitant. The most useful method is that of Folin and Wu in which the precipitating agent is tungstic acid. One of the principal advantages of this method is that a single filtrate may be used for a large number of different analyses.

In the original method the blood is first laked with distilled water, then sodium tungstate added, followed by sulfuric acid. There are numerous modifications and variations of this method. The most commonly used modification is that of Haden in which the sulfuric acid and distilled water are combined and measured as one solution. This dilute acid is added to the blood before the tungstate thereby resulting in conversion of some of the hemoglobin to acid hematin, in which form it is precipitated by the tungstate. It is claimed that this modification will produce filtrates of greater clarity. However, filtrates prepared in this manner may contain as much as 10 per cent more non-protein nitrogen than when the original Folin-Wu procedure is used. This is probably due to the slightly greater solubility of the acid hematin precipitate as compared to the hemoglobin precipitate, the form in which precipitation occurs when the tungstate is added first.

The zinc hydroxide filtrate of Somogyi is also widely used. It is par-

ticularly advantageous in the determination of blood sugar where it gives accurate results. The tungstic acid filtrates may contain substances other than sugar which react with the reagents used in the determination, and thus give results higher than the true sugar values. These substances are removed by the zinc hydroxide method of precipitation. However, filtrates prepared in this manner will give low values for non-protein nitrogen, uric acid and creatinine, since these are precipitated in part by the zinc hydroxide. Consequently if this type of filtrate is used for the determination of sugar, and it is desired to determine any of the other substances on the same sample, it is necessary to prepare a second blood filtrate by the tungstic acid method.

Another commonly used protein precipitant is trichloroacetic acid. It is of great value in those reactions with which tungstic acid would interfere. Common examples of its use as a precipitant are the determinations of calcium, phosphorus, phosphatase, and the sulfonamides.

1. Preparation of Protein-free Blood Filtrate (Folin and Wu).—

1. Reagents.—(a) *Sodium Tungstate*, 10 per cent aqueous solution.

(b) *Sulfuric Acid*, 0.66 N.—To 66 cc. of 1 N sulfuric acid add 33 cc. of distilled water. The 1 N sulfuric acid must be accurately standardized

and will depend upon the number of different determinations desired. About 10 cc. will give sufficient filtrate for all of the routine analyses that can be done on this type of filtrate. Where a single procedure only is desired, 2 cc. of blood suffice, and for two to four procedures, 5 cc. are sufficient.

2. Procedure.—Transfer a measured amount, which is 1 volume, of oxalated blood to a flask having a capacity 15 to 20 times that of the volume taken. Dilute the blood with 7 volumes of distilled water and mix. With a pipet add 1 volume of 10 per cent sodium tungstate and mix. With another pipet add, with constant shaking, 1 volume of 0.66 N sulfuric acid. Close the flask with a rubber stopper and give a few vigorous shakes. If conditions are right hardly a single bubble will form as a result of the shaking.

When blood is properly coagulated, the color of the coagulum gradually changes from pink to chocolate. If this change does not occur, even after standing fifteen to twenty minutes, the coagulation is incomplete due usually to inaccurate 0.66 N solution of sulfuric acid. In such an emergency, the sample may sometimes be saved by the cautious addition of 10 per cent sulfuric acid; add the acid drop by drop, shaking vigorously after each addition and allowing the mixture to stand for a few minutes before adding more, until coagulation is complete.

Pour the mixture on a filter large enough to hold the entire contents of the flask and cover with a watch glass. If the filtration is begun by pouring the first few cubic centimeters of the mixture down the double portion of the filter paper, the filtrate is almost invariably as clear as water from the first drop; if the first portion is not clear, return it to the filter.

On the average, 10 cc. of blood will yield about 35 cc. of filtrate after filtering for thirty minutes; from 45 to 50 cc. in one hour; and about 55 cc. in one and a half hours; while, by allowing the filtration to continue overnight, as much as 65 cc. can be obtained.

The fact that the protein precipitation is done volumetrically makes for several advantages; it not only allows use of all of a small sample of blood, but it gives a filtrate, which regardless of the initial quantity of blood used, is itself 10 per cent blood. Thus, no matter what amount of blood be taken at first, 10 cc. of the filtrate correspond to 1 cc. of blood, 5 cc. of filtrate to 0.5 cc. of blood, and so on. This considerably simplifies the calculations.

The most probable source of error in the above procedure lies in the use of improper sulfuric acid solution. The strength of the sulfuric acid must always be determined, and adjusted if necessary, by titration against an accurate 1 N solution of alkali. If the acid is too weak the filtrate will be cloudy and colored and high values for the non-protein nitrogen and urea will be obtained. If the acid is too strong, a clear and colorless filtrate will be obtained, but the values for sugar and uric acid will be too low.

When the urea nitrogen is to be determined the preparation of the filtrate is modified as follows: To one volume of blood add 7 volumes of distilled water, then add approximately 10 to 20 mg. of a purified urease free from non-protein nitrogen. Allow to stand at room temperature, or in an incubator if the temperature of the room is below 20° C., for twenty minutes. Complete the preparation of the filtrate in the usual manner by the addition of the tungstate and sulfuric acid.

This same filtrate may be used for all other determinations, which use a tungstic acid filtrate, provided no interfering material is present in the added urease. The principal contaminant is non-protein nitrogen, and in some preparations this may exceed the amount to be found in the blood. If this filtrate is to be used for a non-protein nitrogen determination a specially purified urease must be used. Squibb's "Double-strength" urease is one of the few commercial preparations available containing no non-protein nitrogen.

Other extracts may be used for urea if non-protein nitrogen determination is not required. No interference from such a urease preparation has been found to occur with any of the other blood constituents.

II. Determination of Non-protein Nitrogen (Folin and Wu).—

1. *Reagents.*—(a) *Sulfuric-phosphoric Acid Digestion Mixture.*—Mix 300 cc. of phosphoric acid (about 85 per cent H_3PO_4) with 100 cc. of concentrated sulfuric acid. Transfer to a tall cylinder, cover well to exclude ammonia, and set aside for sedimentation of calcium sulfate. This sedimentation is very slow, but in the course of a week or so the top part is clear, and 50 to 100 cc. can be removed by means of a pipet. If this cannot be done, rapid centrifugalization will yield a perfectly clear solution. To 100 cc. of the clear acid mixture add 10 cc. of a 6 per cent copper sulfate solution, and 100 cc. of distilled water.

(b) *Standard Nitrogen Solution.*—A concentrated stock solution is prepared by dissolving 4.716 gm. of pure, dry ammonium sulfate in 1 liter of 0.2 N sulfuric acid. The solution is then diluted to 100 cc. and the solution of this solution 1 cc. contains 0.1 mg. nitrogen.

(c) *Nessler's Solution* (Koch and McMeekin).—Dissolve 22.5 gm. of iodine in 20 cc. of water containing 30 gm. of potassium iodide. After the solution is complete add 30 gm. of pure metallic mercury and shake

the mixture well, keeping it from becoming hot by immersing in tap water from time to time. Continue until the supernatant fluid has lost all color due to iodine. Decant the supernatant from the excess mercury and test for free iodine by adding a few drops thereof to 1 cc. of a 1 per cent solution of starch. If the starch test for iodine is negative the solution may contain mercurous compounds; in this case, add to the remaining solution a few drops of an iodine solution of the same concentration as employed above, until a faint excess of iodine can be detected by the starch solution. Dilute to 200 cc. and mix thoroughly. To 975 cc. of an accurately prepared 10 per cent NaOH solution, now add the entire solution of potassium mercuric iodide prepared above. Mix thoroughly and allow to settle.

2. *Procedure.*—Introduce 5 cc. of the protein-free blood filtrate corresponding to 0.5 cc. of blood, into a dry, 75-cc. test tube graduated at 35 cc. These tubes must be made of pyrex or other resistant glass, and the most convenient size is 200 x 25 mm. Add 1 cc. of the sulfuric-phosphoric acid digestion mixture, and boil vigorously over a micro-burner until the characteristic, dense, acid fumes begin to fill the test tube, which is usually in from three to seven minutes. If the test tube is held in a slightly inclined position, and the heating begun by applying the flame of the micro-burner at the side of the tube and just below the top of the contained mixture, no bumping will occur; as the mixture begins to boil, the flame can be applied lower down and, finally, under the bottom of the tube. Unless this method of heating is followed, bumping is likely to be troublesome, and may even result in the loss of a part or all of the preparation.

When the sulfuric acid fumes are unmistakable, cut down the flame so that the contents of the tube are just visibly boiling and close the mouth of the test tube with a small watch glass.

Continue the heating very gently for two minutes from the time the fumes begin to be unmistakable, even if the solution has become clear and colorless at the end of twenty to forty seconds. If the oxidation is not visibly finished at the end of two minutes, the heating must be continued until the solution is nearly colorless.

Allow the contents to cool for seventy to ninety seconds, and then add 15 to 25 cc. of distilled water; cool further, approximately to room temperature, and add distilled water to the 35-cc. mark. Occasionally there is formed a heavy white precipitate, probably silicates; this may settle out, or can be readily removed by centrifugalizing or filtering after nesslerization, and just before reading against the standard.

When the unknown has been prepared, a standard for comparison is made as follows: Place 3 cc. of the standard nitrogen solution, containing 0.3 mg. of nitrogen, in a 100-cc. volumetric flask; add 2 cc. of the sulfuric-phosphoric acid digestion mixture referred to above, and then about 50 cc. of distilled water.

Then add to the unknown 15 cc. and to the standard 30 cc., respectively, of the Nessler's solution; fill the standard to the mark with distilled water; mix each thoroughly by inverting several times and compare in the colorimeter. It is essential that the unknown and the standard be nesslerized at approximately the same time.

3. *Calculation.*—The reading of the standard, usually 20 mm, multiplied by 30, and divided by the reading of the unknown, gives the non-protein nitrogen in mg. per 100 cc. of blood.

III. Urea Nitrogen (Gentzkow and Masen).—

1. **Reagents.**—(a) *Stock Standard Nitrogen Solution.*—Dissolve 7.074 gm. of ammonium sulfate in 1 liter of 0.1 N sulfuric acid.

(b) *Working Standard Nitrogen Solution.*—Dilute 5 cc. of the above stock solution to 500 cc. with 0.01 N sulfuric acid (0.015 mg. N_2 per cc.).

(c) *Potassium Gluconate*, 1 per cent aqueous solution.—Make up a fresh solution weekly and store in the refrigerator.

(d) *Potassium Persulfate.*—A 2.5 per cent solution of the chemically pure, NITROGEN-FREE salt. Prepare a fresh solution weekly. Keep in the refrigerator and remove only long enough to obtain that required as decomposition is rapid at higher temperatures.

(e) *Urease*, Squibb, Double-strength, Powdered.

(f) *Nessler's Solution* (Folin's), modified by reducing the alkali concentration from 7 per cent to 5 per cent. Transfer 150 gm. of KI and 110 gm. of I_2 to a 500-cc. Florence flask; add 100 cc. of water and 140 to 150 gm. of mercury. Shake continuously and vigorously for seven to fifteen minutes, or until the dissolved iodine has nearly all disappeared. The solution becomes hot. When the solution begins to pale visibly, though still red, cool in running water. Continue shaking until the red has been replaced by the greenish color of the double iodide. This operation should not take more than fifteen minutes. Separate the solution from the surplus mercury by decantation. Dilute the solution to a volume of 2 liters. A proportionately smaller amount may be made. If the cooling was begun in time, the resulting reagent is clear enough for immediate dilution as follows: Place 500 cc. of 10 per cent NaOH in a liter volumetric flask, add 150 cc. of stock Nessler's and dilute to the mark with distilled water. Allow to stand for three days to permit sedimentation of the small amount of precipitate which forms.

(g) *Wratten Gelatin Filter No. 75.*—This filter transmits in the region of wave length 490 $m\mu$. If there is a blue daylight filter in the optical system of the colorimeter, this must be removed.

2. **Procedure.**—Prepare a protein-free blood filtrate as described on page 201. Place 5 cc. of the filtrate in a test tube graduated at 20 and 25 cc. In a similar tube place 5 cc. of the working standard, containing 0.075 mg. of N_2 , and to each add distilled water to the 20-cc. mark. Mix by shaking.

Prepare the nesslerizing solution by mixing 1 part of the gluconate and 1 part of the persulfate solutions. Pour this mixture into an equal volume of Nessler's

mixture must be added to the 20-cc. mark, stopper and mix by vigorous shaking. Allow to stand for fifteen minutes to develop full color, then compare in a colorimeter in the usual manner, using the No. 75 filter over the eyepiece of the colorimeter. Comparisons should be completed within one hour after nesslerization. Keep the tubes stoppered as much of the time as possible.

3. **Calculation.**
$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 15 = \text{mg. urea N per 100 cc. of blood.}$$

1. **Precautions.**—Proportionality holds only over a two-fold range. With a standard equal to 15 mg. urea N per 100 cc., the concentration of the unknown should be not less than 8 nor more than 30 mg. per 100 cc., and

(c) *Standard Creatinine Solution*.—A stock solution is first prepared by dissolving 1.6106 gm. of creatinine zinc chloride in 1 liter of 0.1 N hydrochloric acid. The working standard is prepared by diluting 3 cc. of the stock standard to 500 cc. with 0.01 N hydrochloric acid. Transfer to a bottle and add 4 or 5 drops of toluene or xylene. Five cc. of this solution contain 0.03 mg. of creatinine.

2. *Procedure*.—Place 10 cc. of the protein-free blood filtrate, corresponding to 1 cc. of blood, in a small flask, or test tube. Place 5 cc. of the standard creatinine solution in another flask and add 15 cc. of water. To 25 cc. of the 0.26 per cent solution of picric acid add 5 cc. of 10 per cent sodium hydroxide solution. Then add 5 cc. of the alkaline picrate solution, freshly prepared as above, to the blood filtrate, and 10 cc. to the diluted creatinine standard.

Let stand fifty minutes and compare in the colorimeter. The readings should be completed within sixty minutes from the time the alkaline picrate solution was added to the filtrate and standard.

The creatinine standard solution is so made that 5 cc. contain 0.03 mg. of creatinine, and this amount plus 15 cc. of water represents the standard needed for the vast majority of human bloods, for it covers the range of 1 to 2 mg. per 100 cc. of blood. In the case of bloods showing retention of creatinine take 10 cc. of the standard plus 10 cc. of water, which covers the range from 2 to 4 mg. of creatinine per 100 cc. of blood; or 15 cc. of the standard plus 5 cc. of water, by which 1 to 6 mg. per 100 cc. of blood can be estimated. By taking the full 20 cc. volume of the standard solution, at least 8 mg. can be estimated; but when working with such bloods, it is better to substitute 5 cc. of the blood filtrate plus 5 cc. of water for the usual 10 cc. of filtrate. The concentration of the standard should be within about 1.5 times that of the filtrate in order to obtain proportionality in matching. This is due to the presence of the picric acid. By using the proper filter the effect of the picric acid on the colorimetric matching can be largely eliminated and the proportionality can be extended from a range of 1.5 to 5. The most suitable wave band is 520 $m\mu$. No single filter is available covering this particular wave band, but a combination of two Wratten gelatin filters, No. 45 H plus 58 B2, which may be mounted together between circular cover slips will isolate this wave band effectively. Using such a filter, the range of proportionality will be extended about 4-fold, thus with the 5 cc. standard, equivalent to 1.5 mg. creatinine per 100 cc. of blood it is possible to measure specimens up to about 6 mg. per 100 cc. of blood.

3. *Calculation*.—The reading of the standard, usually 20 mm., multiplied by 1.5, 3, 4.5, or 6 depending upon whether 5, 10, 15 or 20 cc., respectively, of the standard solution were used, and divided by the reading of the unknown, gives the creatinine in mg. per 100 cc. of blood.

When the amount of the blood filtrate available for the creatinine determination is too small to permit a repetition, it is advantageous to start with more than one standard. If, however, a high creatinine should be encountered unexpectedly without several standards ready, the determination can be saved by diluting the unknown with an appropriate amount of the alkaline picrate solution, using for such dilution a picrate solution first diluted with 2 volumes of water, so as to preserve equality between

the standard and the unknown in regard to the concentration of picric acid and sodium hydroxide.

VI. Determination of Uric Acid (Brown).

1. *Reagents.*—(a) *Standard Solution of Uric Acid.*—A stock is first prepared. Transfer exactly 1 gm. of uric acid to a funnel on a 300-cc. Erlenmeyer flask. Transfer 0.45 to 0.5 gm. of lithium carbonate to a 300-cc. beaker, add 150 cc. of distilled water and heat to 60° C., shaking or stirring until all of the carbonate has dissolved. With the hot carbonate solution rinse the uric acid solution into its flask and shake. The uric acid dissolves practically at once. As soon as a clear solution is obtained, cool under running water with shaking and transfer to a liter volumetric flask, rinsing into the flask with distilled water to a volume of 500 cc. Add 25 cc. of formalin, and after shaking to insure thorough mixing, add 3 cc. of glacial acetic acid. Shake to remove most of the carbon dioxide and dilute to the mark. Keep in small, tightly stoppered bottles in a dark place under refrigeration.

(b) *Working Standard.*—Dilute 5 cc. of the stock solution in a liter volumetric flask to 800 cc. with water; add 2 cc. of formalin and 20 cc. of 0.66 N sulfuric acid. Dilute to the mark. Five cc. are equivalent to 0.025 mg. uric acid. This standard keeps about eight weeks.

(c) *Sodium Cyanide Solution.*—Place 5 gm. of sodium cyanide in a 100-cc. volumetric flask and dilute to the mark with distilled water. Prepare fresh every month. *Never pipet this poisonous solution.*

(d) *Uric Acid Reagent.*—Place in a 1000-cc. Erlenmeyer flask 100 gm. of sodium tungstate, 80 cc. of phosphoric acid, and about 700 cc. of water. Boil gently for two hours using a reflux condenser. Cool and dilute to 1 liter.

2. *Procedure.*—Pipet 10 cc. of protein-free blood filtrate into a small Erlenmeyer flask and add 5 cc. of water. To each of two similar flasks add, respectively, 5 and 10 cc. of the uric acid standard, and 10 and 5 cc. of water. To all flasks add from a buret 5 cc. of sodium cyanide solution. Then add 0.5 cc. of the uric acid reagent. Mix and allow to stand for twenty minutes. Compare the unknown in the colorimeter with the standard which it more nearly resembles in depth of color.

3. *Calculation.*—Where the 5 cc. standard containing 0.025 mg. of uric acid is used for the comparison, the reading of the standard times 2.5 divided by the reading of the unknown equals mg. of uric acid per 100 cc. of blood. When the 10 cc. standard is used for the comparison, the reading of the standard times 5 divided by the reading of the unknown equals mg. uric acid per 100 cc. of blood.

VII. Determination of Sugar (Folin and Wu).

1. *Reagents.*—(a) *Stock Sugar Solution and Standards.*—One per cent anhydrous dextrose in 0.25 per cent benzoic acid; this solution, when prepared with 0.25 per cent benzoic acid will not decompose. Two working standards are prepared from this stock standard. The weaker solution contains 0.1 mg. of sugar per cc. and is prepared by diluting 5 cc. of the stock solution to 500 cc. with 0.25 per cent benzoic acid solution. The stronger standard is prepared by diluting 5 cc. of the stock solution to 250 cc. with 0.25 per cent benzoic acid.

(b) *Alkaline Copper Solution.*—Dissolve 40 gm. of pure anhydrous sodium carbonate in about 400 cc. of distilled water in a liter flask. Add

7.5 gm.-of tartaric acid, and when the latter has dissolved, add 4.5 gm. of crystallized copper sulfate. Mix and make up to the liter mark with distilled water.

(c) *Molybdate-Phosphate Solution*.—Place 35 gm. of molybdic acid and 5 gm. of sodium tungstate in a liter beaker. Add 200 cc. of 10 per cent sodium hydroxide solution and 200 cc. of distilled water. Boil vigorously for twenty to forty minutes. Cool, dilute to about 350 cc. with distilled water and add 125 cc. of concentrated (85 per cent) phosphoric acid. Dilute to 500 cc. with distilled water.

2 *Procedure*.—Place 2 cc. of the protein-free blood filtrate, corresponding to 0.2 cc. of blood, in a special blood sugar test tube; these tubes are graduated at 25 cc. and are constricted toward the bottom so as to form a bulb which will contain 4 cc., this amount of fluid rising to just within the constricted portion of the tube. In two other similar tubes place 2 cc. of the standard sugar solutions containing, respectively, 0.2 and 0.4 mg. of dextrose. To each of the three tubes add 2 cc. of the alkaline copper solution.

The surfaces of the mixtures must now have reached the constricted parts of the tubes, and must not lie above these parts. If the bulb of the tube is too large for the volume, 4 cc., a little, but not more than 0.5 cc. of a 1 to 1 aqueous dilution of the alkaline copper solution may be added. Tubes of either too large or too small capacity should be discarded; the surface of the mixture should lie within the constriction.

Transfer the tubes to a boiling water bath, and heat for six minutes; then transfer to a cold water bath and allow to cool, without shaking, for two or three minutes.

Add to each tube 2 cc. of the molybdate-phosphate solution. The cuprous oxide dissolves rather slowly if the amount present is large; but the whole, up to the quantity given by 0.8 mg. of dextrose, dissolves usually within two minutes.

When the cuprous oxide is dissolved, dilute the resulting solutions to the 25-cc. mark with a 1 + 4 dilution of the molybdate-phosphate reagent, mix each tube thoroughly by inverting several times, using care to insure complete mixing, as the greater part of the color is developed in the bulb of the tube. Compare in the colorimeter, using the standard which more nearly approximates the unknown.

3. *Calculation*.—When the weaker standard is used, *i. e.*, the one containing 0.2 mg. of dextrose, the reading of the standard, usually 20 mm., multiplied by 100 and divided by the reading of the unknown, gives the sugar in mg. per 100 cc. of blood. When the stronger standard is used, substitute 200 for the 100 in the calculation.

VIII. Glucose Tolerance Test.

Following the ingestion of a definite amount of glucose, blood sugar is determined at intervals. Urine specimens, collected at the same time intervals, are tested for glucose, and, if positive, the amount present is determined.

The dosage of glucose given by different workers varies considerably. Present practice is to give 100 gm. to all adults of average height and muscular build. This dosage is used rather than one based on weight for it is the liver, pancreas and musculature that are concerned in carbohydrate

metabolism and not the body fat upon which the weight of the individual depends.

For children and for adults who vary widely from the average, a dosage of 1.75 gm. of glucose per kilogram of body weight may be used.

A simplified practice consists of taking a fasting blood specimen and then giving a very heavy carbohydrate breakfast including such items as fruit and cereal, both with large amounts of sugar added, toast with jams or jellies, French toast or hot cakes with syrup, etc. Two hours after this meal, a second blood specimen is taken and a urine specimen is obtained. In a normal individual the blood sugar should approximate the fasting level and there should be no glycosuria.

If there should be any doubt regarding absorption of ingested glucose, resort may be had to intravenous administration. The usual dosage is 50 cc. of a 50 per cent solution of glucose especially prepared for intravenous use.

1. **Reagents.**—Those for blood and urine sugar and in addition:

(a) *Glucose.*—This is usually given in 40 to 50 per cent solution with lemon juice added to make it more palatable.

2. **Procedure.**—Obtain blood and urine specimens from the fasting patient. Give the glucose solution and note the time. Half an hour, one hour, two hours and three hours later take blood and urine specimens. Determine glucose in each blood specimen and test each urine for sugar. If any urine specimens are positive, determine the amount of sugar present.

It is important that the individual be on a full, well-balanced diet for several days prior to the test. If on a much-restricted intake, the results may simulate those of carbohydrate starvation.

3. **Result.**—Record the blood and urine glucose for each specimen and also the dosage of glucose given, with mode of administration.

IX. Determination of Chlorides (Schales and Schales).

1. **Reagents.**—(a) *Standard Sodium Chloride Solution.*—Dry chemically pure sodium chloride at 120° C. to constant weight. Dissolve 0.500 gm. in 200 to 300 cc. of distilled water in a liter volumetric flask and make up to 1 liter with distilled water.

(b) *Mercuric Nitrate Solution.*—Dissolve 1.5 gm. of chemically pure mercuric nitrate in a few hundred cc. of distilled water with the addition of 10 cc. of 10 per cent sodium hydroxide solution, then add distilled water to make 1 liter. The solution should be standardized as the sodium chloride solution.

(c) *Indicator Solution.*—Dissolve 100 mg. of *s*-diphenylcarbazone in 100 cc. of 95 per cent ethyl alcohol and store in a dark bottle in the refrigerator. A fresh solution should be prepared each week.

2. **Procedure.**—Pipet 5 cc. of the Folin-Wu protein-free blood filtrate, corresponding to 0.5 cc. of blood, into a porcelain dish. Add 0.06 cc. (4 drops) of indicator solution, and titrate with the mercuric nitrate solution, using a microburet calibrated in 0.01 cc. intervals. The clear colorless solution turns an intense violet-blue on the addition of the first drop of mercuric nitrate solution in excess.

3. **Calculation.**—The reading of the buret multiplied by 100 gives directly the chloride as sodium chloride in 100 cc. of blood.

X. Determination of Cholesterol (Leiboff).

7.5 gm. of tartaric acid, and when the latter has dissolved, add 4.5 gm. of crystallized copper sulfate. Mix and make up to the liter mark with distilled water.

(c) *Molybdate-Phosphate Solution*.—Place 35 gm. of molybdic acid and 5 gm. of sodium tungstate in a liter beaker. Add 200 cc. of 10 per cent sodium hydroxide solution and 200 cc. of distilled water. Boil vigorously for twenty to forty minutes. Cool, dilute to about 350 cc. with distilled water and add 125 cc. of concentrated (85 per cent) phosphoric acid. Dilute to 500 cc. with distilled water.

2. *Procedure*.—Place 2 cc. of the protein-free blood filtrate, corresponding to 0.2 cc. of blood, in a special blood sugar test tube; these tubes are graduated at 25 cc. and are constricted toward the bottom so as to form a bulb which will contain 4 cc., this amount of fluid rising to just within the constricted portion of the tube. In two other similar tubes place 2 cc. of the standard sugar solutions containing, respectively, 0.2 and 0.4 mg. of dextrose. To each of the three tubes add 2 cc. of the alkaline copper solution.

The surfaces of the mixtures must now have reached the constricted parts of the tubes, and must not lie above these parts. If the bulb of the tube is too large for the volume, 4 cc., a little, but not more than 0.5 cc. of a 1 to 1 aqueous dilution of the alkaline copper solution may be added. Tubes of either too large or too small capacity should be discarded; the surface of the mixture should lie within the constriction.

Transfer the tubes to a boiling water bath, and heat for six minutes; then transfer to a cold water bath and allow to cool, without shaking, for two or three minutes.

Add to each tube 2 cc. of the molybdate-phosphate solution. The cuprous oxide dissolves rather slowly if the amount present is large; but the whole, up to the quantity given by 0.8 mg. of dextrose, dissolves usually within two minutes.

When the cuprous oxide is dissolved, dilute the resulting solutions to the 25-cc. mark with a 1 + 4 dilution of the molybdate-phosphate reagent, mix each tube thoroughly by inverting several times, using care to insure complete mixing, as the greater part of the color is developed in the bulb of the tube. Compare in the colorimeter, using the standard which more nearly approximates the unknown.

3 *Calculation*.—When the weaker standard is used, *i. e.*, the one containing 0.2 mg. of dextrose, the reading of the standard, usually 20 mm., multiplied by 100 and divided by the reading of the unknown, gives the sugar in mg. per 100 cc. of blood. When the stronger standard is used, substitute 200 for the 100 in the calculation.

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metabolism and not the body fat upon which the weight of the individual depends.

For children and for adults who vary widely from the average, a dosage of 1.75 gm. of glucose per kilogram of body weight may be used.

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(a) *Glucose.*—This is usually given in 40 to 50 per cent solution with lemon juice added to make it more palatable

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It is important that the individual be on a full, well-balanced diet for several days prior to the test. If on a much-restricted intake, the results may simulate those of carbohydrate starvation

3. **Result.**—Record the blood and urine glucose for each specimen and also the dosage of glucose given, with mode of administration.

IX. Determination of Chlorides (Schales and Schaless).

1. **Reagents.**—(a) *Standard Sodium Chloride Solution.*—Dry chemically pure sodium chloride at 120° C. to constant weight. Dissolve 0.500 gm. in 200 to 300 cc. of distilled water in a liter volumetric flask and make up to 1 liter with distilled water.

(b) *Mercuric Nitrate Solution.*—Dissolve 1.5 gm of chemically pure mercuric nitrate in a few hundred cc. of distilled water with the addition of 20 cc. of 2 N nitric acid and make up to 1 liter with distilled water. Titrate this solution against 5 cc. of the standard sodium chloride solution, using 4 drops of the indicator, and adjust it to exactly the same strength as the sodium chloride solution.

(c) *Indicator Solution.*—Dissolve 100 mg. of s-diphenylcarbazone in 100 cc of 95 per cent ethyl alcohol and store in a dark bottle in the refrigerator. A fresh solution should be prepared each week.

2. **Procedure.**—Pipet 5 cc. of the Folin-Wu protein-free blood filtrate, corresponding to 0.5 cc. of blood, into a porcelain dish. Add 0.06 cc. (4 drops)⁷ of indicator solution, and titrate with the mercuric nitrate solution, using a microburet calibrated in 0.01 cc. intervals. The clear colorless solution turns an intense violet-blue on the addition of the first drop of mercuric nitrate solution in excess.

3. **Calculation.**—The reading of the buret multiplied by 100 gives directly the chloride as sodium chloride in 100 cc. of blood.

X. Determination of Cholesterol (Leiboff).

1. **Reagents.**—(a) *Standard Solution of Cholesterol in Chloroform.*—A stock solution is first prepared by dissolving 0.160 gm. of pure cholesterol in 100 cc. of chloroform. The working standard is made by diluting 5 cc. of the stock standard to 100 cc. with chloroform. Five cc. of standard contain 0.4 mg. of cholesterol. Preserve in dark-glass bottles, preferably in the refrigerator.

(b) *Acetic Anhydride.*—If not clear and colorless it may be purified by redistillation

(c) *Sulfuric Acid*, concentrated.

(d) *Asbestos Cloth.*—Medium weave cloth is first extracted in a Soxhlet continuous extractor with chloroform to remove impurities. The cloth is then dried and cut into 1 inch squares which are kept in a bottle of ether. The purpose of keeping the asbestos under ether is to prevent loose fiber and powder from falling into the tube when handling, which occurs if the asbestos is dry. B
drying is accomplished h as ether, easy

(e) *Chloroform.*—Chl
preservative. If purification is attempted by redistillation this alcohol is

removed and the chloroform will gradually decompose on standing. The decomposition products will react with the reagents and give erroneous results. It is preferable to use a good grade of anesthetic chloroform rather than attempt purification by redistillation. If the chloroform contains water this may be removed by shaking with anhydrous potassium carbonate, and then filtering.

(f) *Leiboff Extraction Tube* (Fig. 21).—This particular form of tube is no longer available as a stock item from laboratory supply companies, but may be obtained made to specification from Eck and Krebs, New York City.

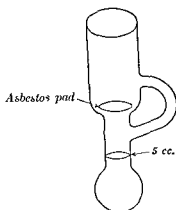


FIG 21 —Leiboff extraction tube.

2. **Procedure.** — Remove a square of the asbestos cloth from the bottle of ether and place in the Leiboff extraction tube as shown in Figure 21. Place the tube in a beaker of hot water until the ether has been driven off. From a pipet drop 0.25 cc. of oxalated blood on to the cloth. Place in a desiccator and allow to dry in an incubator at a temperature not exceeding 40° C. for two hours. If a vacuum pump is available, the desiccator may be evacuated and drying will take place in one hour at room temperature. Introduce about 4 cc. of chloroform into the extraction tube, connect to a reflux condenser and heat by means of a sand bath on a hot plate. Continue the extraction for one hour. Detach the tube, remove the asbestos, allow to cool to room temperature and add chloroform to the 5-cc. mark. In another tube place 5 cc. of the standard cholesterol solution. Prepare a mixture of acetic anhydride and sulfuric acid by adding 0.1 volume of concentrated sulfuric acid to 1 volume of acetic anhydride. Cool in a water bath. This mixture must be freshly prepared and used as soon as cool. To both standard and unknown add 2 cc. of the acetic anhydride-sulfuric acid mixture. Mix and place in a beaker of water at a temperature of 20° to 25° C., and allow to remain in a dark cabinet for thirty minutes.

At the end of this period compare in the colorimeter with the standard set at 15 mm.

3. **Calculation.**—Twenty-four hundred divided by the reading of the unknown equals mg. of cholesterol per 100 cc. of blood.

Caution.—The color developed by the action of the acetic anhydride and sulfuric acid on cholesterol in chloroform solution changes rapidly on removal from the water bath and exposure to light, and since the change in the standard is not at the same rate as in the unknown, it is essential that the matching be made as rapidly as possible, within two minutes from the time that the solutions are removed from the dark cabinet. If a series of unknown are to be read, and this cannot be done in two minutes, it is necessary to prepare another standard. This reaction is also seriously affected by moisture. It is, therefore, essential that all pipets, tubes, colorimeter cups and plungers be absolutely dry throughout the procedure.

In certain bloods, particularly those containing bile pigment, the shade of color in the unknown may be quite different from that in the standard and difficulty will be experienced in the colorimetric matching. This difficulty can be eliminated by using in the eyepiece of the colorimeter a red filter such as Wratten No. 71-A.

XI. Determination of Cholesterol: Free, Esters, and Total (Schoenheimer and Sperry).

Free cholesterol is precipitated with digitonin and the digitonide so obtained is determined colorimetrically by means of a modified Lieberman-Burchard reaction. For total cholesterol, a portion of the serum extract is first hydrolyzed with alkali, converting the cholesterol ester present to free cholesterol. This is then determined in the same manner as the free cholesterol. Total cholesterol minus free cholesterol equals cholesterol ester. Because the cholesterol is first isolated from interfering material, this method is probably more accurate and specific than other methods commonly used in which direct determination without previous isolation is used. Also the color is developed in glacial acetic acid instead of chloroform as in the original Lieberman-Burchard reaction, and in glacial acetic acid the stability of the color is much greater resulting in greater accuracy in the colorimetric measurements.

1. **Reagents.**—(a) *Digitonin Solution.*—Dissolve 1 gm. of digitonin in 1 liter of distilled water. Place the solution in the refrigerator for several days. Remove the precipitate that forms by filtration. Concentrate the solution to approximately one-half. This is best done by placing in a 1 liter bottle and marking the bottle at a point representing one-half the total volume. Insert a two-hole stopper containing inlet and outlet tubes in the mouth of the bottle. The mouth of the inlet tube should be kept about 2 cm. above the surface of the solution. A rapid current of air filtered through cotton is blown or drawn by suction through the bottle which is immersed in boiling water. Water is removed until the level of the solution falls to the half-way mark. This concentration requires three to four hours. Should a sediment appear in the concentrated solution, filter.

(b) *Acetone-Absolute Alcohol.*—Equal parts of each.

(c) *Acetone-Ether.*—One part of ether plus 2 parts of acetone.

(d) *Potassium Hydroxide Solution.*—10 gm. in 20 cc. of water.

(e) *Hydrochloric Acid Solution.*—Dilute 15 cc. of concentrated HCl to

100 cc. with distilled water. (Approximately 5 per cent solution of hydrogen chloride.)

(f) *Cholesterol Stock Solution*.—Dissolve 200 mg. of cholesterol in 100 cc. of glacial acetic acid.

(g) *Cholesterol Working Standard*.—Dilute 5 cc. of the stock solution to 100 cc. with glacial acetic acid.

2. *Procedure*.—(a) *Preparation of Acetone-Alcohol Extract*.—Hold a 20-cc. flask over a Bunsen burner flame to approach the boiling-point of the hand. Lower a pipet containing 1 cc. of serum into the flask so that the tip is within a few millimeters of the hot solvent. Rocking the flask and pipet, slowly introduce the serum. Agitate the mixture vigorously to disintegrate the clumps of protein. Heat the contents of the flask to boiling on the water bath. Cool, bring to volume with acetone-alcohol, mix thoroughly and pass through a dry filter to obtain a clear extract.

(b) *Precipitation of Free Cholesterol*.—To 6 cc. of the extract in a 15-cc. centrifuge tube add 3 cc. of digitonin solution. Stir with a fine glass rod and leave it in the tube. Place the tube in a pint- or quart-size Mason jar, cover tightly and leave at room temperature for at least one hour, preferably overnight.

Stir gently to free particles from the walls of the tube. Remove the stirring rod without touching the upper part of the tube and lay it on a rack so that no adherent precipitate is rubbed off. Centrifugalize at 2500 r.p.m. for fifteen minutes. Slowly aspirate the supernatant fluid with a capillary pipet to which suction is very cautiously applied. Do not touch the walls nor disturb the precipitate. A few particles, probably cholesterol ester, usually float at or near the surface and are removed with the fluid.

Replace the stirring rod. Wash the walls and rod with about 3 cc. of acetone-ether. After thorough stirring, transfer the rod to the rack and centrifugalize the tube for five minutes. Aspirate the supernatant fluid employing less suction than with the acetone-alcohol. For the two succeeding washings use ether only. Finally place the stirring rod in the tube and heat in a water bath at approximately 40° C. to dry the precipitate (two to three minutes). The last traces of ether are drawn off with the aid of a pipet attached to a suction pump.

(c) *Precipitation of Total Cholesterol*.—To 2 cc. of extract in a 15-cc. centrifuge tube, add 2 drops of the KOH solution. Stir into solution and leave the tube in the tube. Set aside in a Mason jar containing a layer of sand about 3 cm. deep which has been heated to 40° C. Clamp the cover on and keep the jar at 37° to 40° C. for thirty minutes. This procedure hydrolyzes the cholesterol esters.

After cooling the tube to room temperature, add 1 cc. of the acetone-alcohol mixture and titrate with 5 per cent HCl, using phenolphthalein as the indicator. Stir after the addition of each drop of acid. Since alkali interferes with the precipitation of cholesterol digitonide, an excess of HCl is necessary. A slight excess of the latter does not affect the precipitation.

Add 2 cc. of digitonin solution, stir thoroughly and set aside at least one hour, preferably overnight. Centrifugalize and wash the precipitate as for free cholesterol. Only one ether washing is necessary.

(d) *Color Development and Measurement.*—Add to each tube 2 cc. of glacial acetic acid, washing down the walls of the tube to catch adherent particles of cholesterol digitonide. Dissolve by warming the tube in a water bath at 50° to 60° C. and stirring. Adjust the bath to 25° C. and allow the tubes to come to temperature equilibrium (thirty seconds). Remove the tubes one at a time and add 4 cc. of acetic anhydride followed by 0.2 cc. of concentrated sulfuric acid. Stir vigorously, then place the tube in a water bath maintained at 25° C. in complete darkness.

Simultaneously prepare a standard using 2 cc. of the cholesterol standard, 4 cc. of acetic anhydride and 0.2 cc. of sulfuric acid.

Make the color comparisons between twenty-seven and thirty-seven minutes after addition of the acid. When using a visual colorimeter place over the ocular a red color filter such as Wratten color filter No. 71A (Eastman Kodak Company). With a photoelectric colorimeter any of the red glass filters usually furnished with the instrument, and having maximum transmission between 620 to 660 $m\mu$ may be used.

3. Calculations.—(a) *For the Visual Colorimeter:*

$$(1) \text{ Free cholesterol in mg. per 100 cc. serum} = \frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 66.7$$

$$(2) \text{ Total cholesterol in mg. per 100 cc. serum} = \frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 200$$

$$(3) \text{ Total cholesterol} - \text{Free} = \text{Cholesterol esters.}$$

(b) *For the Photoelectric Colorimeter:*

$$(1) \text{ Free cholesterol in mg. per 100 cc. serum} = \frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 66.7$$

$$(2) \text{ Total cholesterol in mg. per 100 cc. serum} = \frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 200$$

$$(3) \text{ Total cholesterol} - \text{Free} = \text{Cholesterol esters.}$$

If the scale of the photoelectric colorimeter is calibrated to read per cent transmission, the density value is obtained by subtracting the logarithm of the transmission reading from 2.

XII. Icterus Index (Bernheim).

1. *Reagents.*—(a) *Potassium Dichromate, 1 to 10,000 Solution.*—Dissolve 0.1 gm. of potassium dichromate in about 500 cc. of distilled water in a liter volumetric flask. Add 2 drops of concentrated sulfuric acid and dilute to the mark with distilled water. Preserve in a dark-glass bottle.

(b) *Sodium Chloride Solution, 0.85 or 0.9 per cent.*

2. *Procedure.*—The standard 1 to 10,000 solution of potassium dichromate is placed in one colorimeter cup which is set at 15 mm. The clear serum is placed in the other colorimeter cup and matched against the standard. If the color of the serum is too deep it is accurately diluted with 0.9 per cent sodium chloride solution, the dilution being 1 to 2 or 1 to 5 or greater, if required. The ease of colorimetric matching may be improved by using a deep-blue glass, or Wratten gelatin filter in the eyepiece of the colorimeter.

3. *Calculation.*—The reading of the standard, 15 mm., multiplied by the dilution of the serum, if any, and divided by the reading of the serum gives the icterus index.

NOTE.—Carrots in the diet impart a yellow color to the blood serum which gives a high index. No carrots should be eaten the day preceding the test and the blood should be drawn before breakfast to avoid chyle. Since this test is simply a measurement of the color of the serum, it is evident that even the slightest trace of hemolysis will vitiate the test and it is of utmost importance that hemolysis be avoided. The needle and syringe used should be entirely dry. The blood is allowed to clot for at least one hour in a dry centrifuge tube, protected from the light, and then centrifugalized so as to obtain a clear serum.

XIII. Quantitative Determination of Bilirubin (Evelyn and Malloy).

Since the yellow color of the serum is due to the bilirubin, a measurement of the icterus index will give a rough estimate of the amount of bilirubin present. However certain inaccuracies result due to the presence of other yellow pigments and the more specific reaction of van den Bergh is preferable. No satisfactory method for the quantitative determination of bilirubin by the van den Bergh reaction utilizing a visual colorimeter has been found. The method of Evelyn and Malloy is accurate and convenient, and where a photoelectric colorimeter is available this is the method of choice. It cannot be used with a visual colorimeter, because of the presence of turbidity. With the photoelectric instrument the effect of this turbidity can be eliminated by the use of a blank. The method described herewith is to be used only with a photoelectric colorimeter. The quantitative van den Bergh reaction is described on page 69.

1. **Reagents.**—(a) *Sulfanilic Acid Solution.*—Dissolve 1 gm. of sulfanilic acid in 15 cc. of concentrated hydrochloric acid and dilute to 1 liter with distilled water.

(b) *Sodium Nitrite*, 0.5 per cent solution.—Preserve in a dark-glass bottle in the refrigerator.

(c) *Diazo Reagent.*—Add 0.3 cc. of the sodium nitrite solution to 10 cc. of the sulfanilic acid solution. Prepare freshly as required.

(d) *Diazo Blank.*—Dilute 15 cc. of concentrated hydrochloric acid to 1 liter with distilled water.

(e) *Methyl Alcohol*, absolute.

2. **Procedure.**—All solutions must be added in the exact order as given. Dilute 1 cc. of serum or plasma to 10 cc. with distilled water. To the sample tube add 5 cc. of methyl alcohol, 1 cc. of diazo reagent and 4 cc. of diluted serum. To the blank tube add 5 cc. of methyl alcohol, 1 cc. of diazo blank, 4 cc. of diluted serum.

Each tube is mixed gently by inversion after addition of the serum. Care must be taken to handle both tubes in exactly the same manner so that any turbidity which may result will be the same in each. If bubbles

Use a color filter transmitting wave band 540 mμ. Insert the sample tube and record the reading of the galvanometer. If the galvanometer reading is below 10, add 10 cc. of 50 per cent methyl alcohol to each tube and read again. Multiply the answer so obtained by 2.

3. **Calculation.**
$$\frac{2 - \log \text{galvanometer reading}}{K} \times 100 = \text{mg. bilirubin}$$
 per 100 cc. of serum or plasma. K is a constant obtained with pure solu-

tions of bilirubin and for the Evelyn instrument this value is 6.72. When the Evelyn colorimeter is used this value may be substituted in the above equation, but if a different colorimeter is used this value must be determined in the following manner:

Dissolve 10 mg. of pure bilirubin, accurately weighed, in 100 cc. of chloroform. A portion of this solution is diluted to a final concentration of 0.01 mg. per cc. with ethyl alcohol. In a series of colorimeter tubes each containing 1 cc. of diazo reagent, place 1, 2, 4, and 8 cc. of the bilirubin solution in ethyl alcohol. Add to each tube sufficient ethyl alcohol to make the final volume 10 cc. Prepare a blank tube with 1 cc. of the diazo blank plus 9 cc. of ethyl alcohol. The above amounts are equivalent to

the 2 - log values are obtained. Each of these values are divided by their respective concentrations of bilirubin in terms of mg. per 100 cc. The average of these values represents K for the particular instrument used.

XIV. Determination of Inorganic Phosphorus (Fiske and Subbarow).

1. *Reagents.*—(a) *Sulfuric Acid, 10 N.*—Four hundred and fifty cc. of concentrated sulfuric acid added to 1300 cc. of water.

(b) *Molybdic Acid Solution.*—Dissolve 25 gm. of ammonium molybdate in 200 cc. of water. Rinse into a liter volumetric flask containing 300 cc. of 10 N sulfuric acid. Dilute to the mark with water and mix.

(c) *Trichloroacetic Acid, 10 per cent solution.*

(d) *Standard Phosphate Solution.*—A stock standard is prepared by dissolving 0.3509 gm. of pure monopotassium phosphate (KH_2PO_4) in 1 liter of distilled water. Add 10 cc. of chloroform as a preservative and keep in the refrigerator. To prepare the working standard transfer 10 cc. of the stock standard to a 100-cc. volumetric flask, add 80 cc. of trichloroacetic acid and dilute to the mark with distilled water. Five cc. equal 0.01 mg. of phosphorus.

(e) *Amino-naphthol-sulfonic Acid Reagent.*—Dissolve 30 gm. of sodium bisulfite (NaHSO_3) and 1 gm. of crystalline sodium sulfite ($\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$) or 0.5 gm. of anhydrous sodium sulfite, in 200 cc. of distilled water. Add 0.5 gm. of purified 1, 2, 4-amino-naphthol-sulfonic acid and stir thoroughly. Preserve in a dark-glass bottle. This reagent should be prepared freshly once a month. The sediment that forms will settle to the bottom and need not be filtered out if care is taken not to stir it up.

2. *Procedure.*—Either serum or plasma is used in the determination, and there should be no hemolysis present. Transfer 5 cc. of trichloroacetic acid to a small Erlenmeyer flask. While the flask is being gently rotated, run in 2 cc. of the serum or plasma from an accurate pipet. Close the mouth of the flask with a rubber stopper and shake vigorously a few times. Filter through an ashless filter such as Whatman No. 42.

Transfer 5 cc. of the filtrate to a tube graduated at 10 cc. Into a similar tube measure 5 cc. of the standard phosphate solution. To both tubes add 1 cc. of the molybdic acid reagent and 0.1 cc. of the sulfonic acid reagent. Dilute to the mark with distilled water, mix thoroughly, allow to stand ten minutes and compare in the colorimeter.

3. **Calculation.**—The reading of the standard, usually 20 mm., multiplied by 4 and divided by the reading of the unknown equals mg. of inorganic phosphorus per 100 cc. of serum or plasma.

XV. **Determination of Serum Alkaline and Acid Phosphatase** (Shinowara, Jones, and Reinhart).

(b) *Acetic Acid*, 1 N, standardized.

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layer to the 100-cc. mark. Mix. Keep in the refrigerator.

(d) *Alkaline Phosphatase Substrate.*—In a 100-cc. glass-stoppered mixing cylinder mix 3 cc. of petroleum ether, 50 cc. of the stock buffered glycerophosphate, and 2.8 cc. of 0.1 N sodium hydroxide. Dilute the aqueous layer to the 100-cc. mark and mix. Keep in the refrigerator. The pH of this solution should be 10.9 within 0.1.

(e) *Acid Phosphatase Substrate.*—To a 100-cc. glass-stoppered mixing cylinder add 3 cc. of petroleum ether, 50 cc. of stock buffered glycerophosphate, and 5 cc. of 1 N acetic acid. Dilute the aqueous layer to the 100-cc. mark and mix. Keep in the refrigerator. The pH of this solution should be approximately 5.

(f) *Trichloroacetic Acid*, 30 per cent solution.

2. **Procedure.**—(a) *Alkaline Phosphatase.*—Measure exactly 9 cc. of the alkaline phosphatase substrate into a test tube. Warm to 38° C., add 1 cc. of serum and place in a water bath at 38° C. for exactly one hour. Then add immediately 2 cc. of 30 per cent trichloroacetic acid. Mix and filter through Whatman No. 42 filter paper. Label this the test sample.

A control sample is prepared at or near the end of the sixty minute incubation period by adding 2 cc. of 30 per cent trichloroacetic acid to 9 cc. of the alkaline phosphatase substrate, followed by 1 cc. of the serum. Filter through Whatman No. 42 filter paper.

Measure 8 cc. of each of the filtrates into 10-cc. graduated cylinders and proceed from this point according to the method of Fiske and Subbarow for inorganic phosphorus, page 215.

(b) *Acid Phosphatase.*—Follow the method exactly as under alkaline phosphatase except that the acid phosphatase substrate is substituted for the alkaline.

3. **Calculation.**—For either method: Reading of the standard multiplied by 6 and divided by the reading of the unknown equals mg. inorganic phosphorus per 100 cc. of serum. In the case of alkaline phosphatase, the amount of inorganic phosphorus per 100 cc. in the control sample subtracted from the amount in the test sample equals phosphatase activity in modified Bodansky units. For acid phosphatase the difference in phosphorus content between test sample and control sample equals phosphatase activity in Shinowara-Jones-Reinhart units.

NOTE.—In the above method the normal values for alkaline phosphatase activity are somewhat higher than those given for the Bodansky method. This higher value is due to the higher pH value at which phosphatase

activity is determined. Normal values by this method have been found to range from 2 to 9 units, but are higher in children.

Normals for acid phosphatase activity by the above method have been found to range from 0 to 1.1 units.

XVI. Determination of Calcium (Roe and Kahn).

1. Reagents.—In addition to the solutions required for phosphorus determinations, the following are required:

(a) *Standard Calcium Solution.*—Prepare a stock solution by dissolving 0.4991 gm. of pure calcium carbonate in about 50 cc. of 10 per cent trichloroacetic acid in a 1000-cc. volumetric flask. Iceland spar is preferred, if available. Shake well, and when evolution of CO_2 has ceased, dilute to the mark with 10 per cent trichloroacetic acid. To prepare the working standard transfer 10 cc. of the stock solution to a 100-cc. volumetric flask, add 70 cc. of 10 per cent trichloroacetic acid and dilute to the mark with distilled water. Five cc. equal 0.1 mg. of calcium.

(b) *Alkaline Alcohol Wash Reagent.*—In a 100-cc. cylinder place 58 cc. of 95 per cent ethyl alcohol, add 10 cc. of amyl alcohol and make up to 100 cc. with distilled water. Add 2 drops of 1 per cent phenolphthalein and 5 per cent sodium hydroxide, a drop at a time, with repeated shaking until a distinct pink is obtained.

(c) *Sodium Hydroxide*, 25 per cent solution.

(d) *Trisodium Phosphate*, Na_3PO_4 , 5 per cent solution.

2. Procedure.—Two cc. of serum are precipitated with 8 cc. of 10 per cent trichloroacetic acid in the same manner as in the phosphorus determination.

Where phosphorus and calcium are to be determined on the same specimen, sufficient filtrate for both determinations may be obtained by precipitating 3 cc. of serum with 12 cc. of the trichloroacetic acid.

To a graduated centrifuge tube, transfer 5 cc. of the filtrate and to another similar tube transfer 5 cc. of the standard calcium solution. The tips of the centrifuge tubes used must be sufficiently narrow so that the diameter at the 0.1 cc. mark will not exceed 7 mm. but must not be too finely drawn out. They must be absolutely clean and when not in use should be kept immersed in the dichromate-sulfuric acid cleaning solution. No reliance should be placed on the graduation marks, as they have been found to be inaccurate in many tubes and the 10-cc. mark should be checked. If found inaccurate, a new mark should be made. To both tubes add 1 cc. of 25 per cent NaOH , mix by twirling and allow to stand for five minutes. Then add 1 cc. of the trisodium phosphate solution and allow to stand for one hour to complete precipitation of the calcium phosphate.

Centrifugalize for two minutes. Decant the supernatant fluid with our smooth movement that will not disturb the precipitate. With the mouth still inverted, the tube is placed upon a clean filter paper and allowed to

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from a pipet about 3 cc. of alkaline alcohol wash reagent in such a manner as to break up the mat of $\text{Ca}_3(\text{PO}_4)_2$ in the bottom of the tube. This is done by using a bulb pipet with a fine tip and blowing forcefully, directing the stream upon the calcium phosphate precipitate. If the mat is not broken up completely by this procedure, it must be fragmented thoroughly

with a clean glass stirring rod. The walls of the tube are now washed down with an additional 2 cc. of the alkaline alcohol wash reagent. The tubes are centrifugalized again for two minutes, then decanted and drained as above.

Redissolve the precipitate in both standard and unknown in 4 cc. of 10 per cent trichloroacetic acid, add to each tube 1 cc. of the molybdic acid reagent and 0.4 cc. of the sulfonic acid reagent, the same as are used in the phosphorus determination. Dilute to the 10-cc. mark with distilled water, mix, allow to stand ten minutes, then compare in the colorimeter.

3. Calculation.—The reading of the standard multiplied by 10 and divided by the reading of the unknown equals the mg. of calcium per 100 cc. of blood.

Caution.—This reaction is a reaction for phosphorus based upon the amount of phosphorus contained in the calcium phosphate precipitate. It has been found impossible to obtain reagents which are absolutely calcium-free or free of other substances which would also give color. Therefore, a standard calcium solution is prepared and treated in the same manner throughout as the blood filtrate. Consequently, any error produced in the blood calcium determination as a result of color-producing substances in the reagents used will be exactly balanced by a similar error in the standard used, provided the standard is treated in the same manner as the unknown and with the same reagents. Most filter paper contains traces of calcium. It is, therefore, necessary to use calcium-free filter paper in filtering the proteins from the blood. A double acid-washed paper, such as Whatman No. 42, has been found to meet these requirements. As a result of the action of the 25 per cent NaOH and 5 per cent trisodium phosphate on the glass bottles, a precipitate of silicates will form which will give a color. If kept in tall bottles, the precipitate will settle to the bottom, and the clear supernatant fluid can then be removed with a pipet; otherwise, it is necessary to filter both solutions just before use.

In previous methods calcium was determined by precipitation of the oxalate from the diluted blood serum without prior removal of the blood serum proteins. It has been found that when serum is so precipitated, about 5 to 15 per cent of the calcium fails to be precipitated, consequently when the precipitation is carried out on the protein-free trichloroacetic filtrate, the results will average about 10 per cent higher than those usually published as normal based upon the usual precipitation from the blood serum in the presence of the proteins. The method herein described gives results similar to those obtained with the oxalate precipitation on the protein-free trichloroacetic filtrate. The normals are consequently higher than usually published, being 10 to 12 mg. per 100 cc. of blood instead of 9 to 11 mg.

XVII. Hemoglobin by the Determination of Iron (Wong).

1. Reagents.—All reagents except the standard must be iron-free as shown by the blank tests.

(a) *Sulfuric Acid*, concentrated.

(b) *Sodium Tungstate*, 10 per cent solution.

(c) *Potassium Persulfate*, saturated solution. Introduce about 7 gm. of pure potassium persulfate into a small glass-stoppered bottle and shake with 100 cc. of distilled water. The undissolved crystals settle and later

dissolve in part to replace persulfate lost by decomposition at room temperature.

(d) *Potassium Thiocyanate*.—Prepare an approximately 3 N solution by dissolving 146 gm. of pure potassium thiocyanate in distilled water and making up to a volume of 500 cc. Filter if necessary. Add 20 cc. of pure acetone as a preservative.

(e) *Standard Iron Solution*.—Weigh out exactly 0.861 gm. of crystallized ferric ammonium sulfate and dissolve it in 50 cc. of distilled water. Add 20 cc. of 10 per cent iron-free sulfuric acid. Dilute to 1 liter. Each cc. will contain 0.1 mg. of iron for use as a regular standard. To make weaker standards, dilute this solution accordingly.

2. *Procedure*.—Transfer exactly 0.5 cc. of blood to a 50-cc. volumetric flask and add 2 cc. of iron-free concentrated sulfuric acid. Whirl the flask for a few minutes. Add 2 cc. of potassium persulfate solution and mix. Dilute to about 25 cc. with distilled water, add 2 cc. of the sodium tungstate solution. Mix. Cool to room temperature under the tap, and dilute to 50 cc. with distilled water. Stopper the flask and invert several times to insure thorough mixing. Filter through a dry filter paper. Pipet exactly 20 cc. of filtrate into a test tube graduated at 20 and 25 cc.

Measure exactly 1 cc. of the standard iron solution, containing 0.1 mg. of iron, into another similar tube, add 0.8 cc. of iron-free concentrated sulfuric acid, and dilute to the 20-cc. mark. Cool to room temperature under the tap.

Now add to both the unknown and the standard 1 cc. of saturated potassium persulfate and 1 cc. of the 3 N potassium thiocyanate solution. Mix, and compare in the colorimeter.

3. *Calculation*.—Since the 20 cc. of filtrate taken represents 0.2 cc. of whole blood and the standard solution contains 0.1 mg. of iron, the reading of the standard, usually 20 mm., multiplied by 50 and divided by the reading of the unknown, gives the mg. of iron per 100 cc. of blood.

This figure divided by 3.35 equals the hemoglobin expressed as gm. per 100 cc. of blood.

NOTE.—If it is impossible to secure reagents, especially sodium tungstate, which are absolutely iron-free, the standard may be run in exactly the same manner as the test. For this purpose, take 2.5 cc. of the standard iron solution, add 2 cc. of the sulfuric acid, 2 cc. of the potassium persulfate, dilute to about 25 cc., and add 2 cc. of the sodium tungstate solution. Cool and dilute to 50 cc. Of this final solution, use 20 cc. as the standard, since it contains exactly 0.1 mg. of iron, and add to it and the unknown 1 cc. of saturated potassium persulfate and 1 cc. of the 3 N KCNS solution.

In this manner, due allowance is made for all iron contained in the reagents, without the necessity of running blank determinations.

It is particularly important to use ash-free filter paper, such as Whatman No. 12 or 40, as ordinary filter paper may contain appreciable quantities of iron.

XVIII Determination of Sulfonamides (Bratton and Marshall)

A. *In Blood*.—1. *Reagents*.—(a) *Trichloroacetic Acid*, 15 per cent solution.

(b) *Sodium Nitrate*, 0.1 per cent solution, prepared each day.

(c) *Ammonium Sulfate*, 0.5 per cent solution.

(d) *N*-(1-naphthyl)ethylenediamine Dihydrochloride, 0.1 per cent solution.—Stored in a dark bottle in the refrigerator this solution will keep for one week.

(e) *Hydrochloric Acid*, 4 N solution.

(f) *Standard Sulfonamide Solutions*.—A stock standard solution con-

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Use the dry powdered drug (U.S.P. or C.P.) not the tablets which contain some inert matter. These stock standards are stable for several months if kept under refrigeration. From the stock standard solutions three working standards are prepared. To 10 cc., 5 cc., and 2 cc., of the stock standard contained in 100-cc. volumetric flasks add 18 cc. of the 15 per cent trichloroacetic acid solution and then make up to volume with distilled water. These standards contain 1, 0.5 and 0.2 mg. of the drug per 100 cc. of solution.

2. *Procedure*.—(a) *Free Drug*.—Dilute 2 cc. of oxalated blood in a flask with 30 cc. of distilled water. After five minutes add 8 cc. of 15 per cent trichloroacetic acid, shaking well. Filter out the precipitate. To 10 cc. of the filtrate and 10 cc. portions of each of the three working standard solutions add 1 cc. of sodium nitrite solution. After three minutes, add 1 cc. of ammonium sulfamate solution to each, shaking well to expel bubbles. After two more minutes, add 1 cc. of *N*-(1-naphthyl)ethylenediamine dihydrochloride solution. Allow to stand in the dark ten minutes and then read in the colorimeter against the standard which most nearly approximates the unknown in color.

(b) *Total Drug*.—Treat 10 cc. of the filtrate prepared as above with 0.5 cc. of 4 N hydrochloric acid in a boiling water bath for one hour. Treat 10 cc. of each working standard in the same manner. Cool and adjust volume to 10 cc. Subsequent procedure is the same as for the free drug.

3. *Calculation*.—The reading of the standard times the factor corresponding to the standard used, divided by the reading of the unknown equals mg. of sulfonamide drug per 100 cc. of blood. The factor for the 1 mg. per 100 cc. standard is 20, for the 0.5 mg. standard is 10, and for the 0.2 mg. standard is 4.

To correct for the drug lost in precipitation of proteins with trichloroacetic acid, multiply the results obtained above by the following corrections.

	<i>Free Drug</i>	<i>Total Drug</i>
Sulfanilamide	No corrections necessary	
Sulfaguanidine	No corrections necessary	
Sulfapyridine		
(Less than 5 mg./100 cc.)	No corrections necessary	
Sulfapyridine		
(More than 5 mg./100 cc.)	1 1	1 1
Sulfathiazole	1 1	

For the conjugated sulfathiazole subtract the uncorrected free from the uncorrected total and multiply the difference by 1.3.

B. *In Urine*.—1. *Reagents*.—The same as those used for blood.

2. *Procedure*.—Dilute albumin-free urine so that it will contain about 1 to 2 mg. of sulfonamide per 100 cc. To 50 cc. of this diluted urine in a

100-cc. volumetric flask add 5 cc. of 4 N hydrochloric acid and dilute to the mark.

Treat 10 cc. of this second dilution for free sulfonamides in exactly the same manner as blood filtrate is treated above. Heat a second 10-cc. portion, without adding further acid, as for total sulfonamide.

If the urine contains albumin, dilute it and then follow the procedure as given for whole blood above.

3. **Calculations.**—Calculate as for blood, making due allowance for the urine dilution factor.

XIX. Blood Proteins (Unpublished method, Army Medical School).

A. **Determination of Serum Proteins.**—Ordinarily it is the serum proteins which are determined, that is, albumin and globulin. The method below determines total protein and albumin directly, the globulin being found by difference. When it becomes necessary to determine fibrinogen, oxalated plasma must be used. The fibrinogen is precipitated as fibrin and separated from the plasma solution. It is then determined directly as given under fibrinogen, page 222.

1. **Reagents.**—(a) *Standard Nitrogen Solution.*—Prepared as for non-protein nitrogen determination, page 201. One cc. contains 0.1 mg. of nitrogen.

(b) *Nessler's Solution.*—Prepared as for non-protein nitrogen determination, page 201.

(c) *Sulfuric Acid-Selenium Digestion Mixture.*—Add 0.2 gm. of metallic selenium to 100 cc. of concentrated sulfuric acid in a beaker. Heat gently until the selenium dissolves. Do not overheat or selenium may be volatilized.

(d) *Sodium Sulfate, 22.5 per cent.*—Place exactly 22.5 gm. of anhydrous sodium sulfate in a 100-cc. volumetric flask. Add distilled water to the bottom of the neck of the flask. Heat in a beaker of hot water and swirl until all the salt has dissolved. Place in a 37° to 38° C. incubator. After the temperature of the solution has reached the temperature of the incubator, dilute to the 100-cc. mark. Since this solution is supersaturated at room temperature, it must be kept in the incubator to prevent crystallization of the salt.

(e) *Sodium Tartrate, 2 per cent solution.*

(f) *Sodium Chloride, 0.9 per cent solution.*

(g) *Trichloroacetic Acid, 5 per cent solution.*

2. **Procedure.**—(a) *Total Serum Proteins.*—Dilute 1 cc. of serum to 50 cc. with 0.9 per cent NaCl. Transfer 1 cc. of the diluted serum to a pyrex digestion tube, 25 x 200 mm., graduated at 38 and 50 cc. Add 0.5 cc. of sulfuric acid-selenium digestion mixture and 0.5 cc. of the sodium sulfate of a microburner the tube is filled th a small watch glass or conical funnel and reduce the heat until the acid is barely boiling. Continue heating until the mixture becomes clear and colorless after about five to ten minutes. Let the mixture cool for several minutes, then add about 30 cc. of distilled water and 1 cc. of 2 per cent sodium tartrate. Cool to room temperature by immersion in a beaker of water, then dilute to the 38-cc. mark and add Nessler's solution to the 50-cc. mark. Mix immediately, and compare colorimetrically with a standard nitrogen solu-

tion. Note that only 12 cc. of Nessler's solution are used. It has been found that the stability of color is greater when this amount is used.

Prepare the standard as follows: Place 2 cc. of standard nitrogen solution in a similar digestion tube, add 0.5 cc. of sodium sulfate solution and digest with 0.5 cc. of the digestion mixture in the same manner as for the protein. Cool, dilute, and nesslerize similarly.

In all comparisons of total nitrogen, albumin, and NPN, use a filter transmitting 490 $m\mu$ with the photoelectric colorimeter and Wratten No. 75 with the visual instrument.

(b) *Albumin*.—To 0.5 cc. of serum, add 9.5 cc. of 22.5 per cent sodium sulfate. Incubate at 37° to 38° C., for at least three hours, or preferably overnight. Place the funnel, collecting flask, watch glass, and filter paper in the incubator at the same time to allow them to come to incubator temperature. Filter the serum through a Whatman No. 50 filter paper, returning the first portions of the filtrate (if not clear) to the filter. Keep the funnel covered with a watch glass to prevent evaporation. Pipet 0.5 cc. of the filtrate into a digestion tube and treat in the same manner as for total nitrogen, except that no more sodium sulfate is added.

(c) *Non-protein Nitrogen*.—Dilute 1 cc. of serum to 10 cc. with 5 per cent trichloroacetic acid. Filter. Transfer 5 cc. of filtrate to a digestion tube. Digest, nesslerize, and compare in the colorimeter with the standard in the same manner as for total nitrogen.

3. *Calculations*.—(a) *Total Nitrogen* (Protein and Non-protein).

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} = \text{gm. N per 100 cc. of serum}$$

$$\text{Total Protein} = (\text{Total Nitrogen} - \text{NPN}) \times 6.25$$

(b) *Albumin Nitrogen plus Non-protein Nitrogen*.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.8 = \text{gm. albumin N plus NPN per 100 cc. of serum}$$

$$\text{Albumin} = [(\text{Albumin N} + \text{NPN}) - \text{NPN}] \times 6.25$$

(c) *Non-protein Nitrogen*.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.04 = \text{gm. NPN per 100 cc. serum}$$

(d) *Globulin*.

$$\text{Globulin} = \text{Total Protein} - \text{Albumin}.$$

The non-protein nitrogen normally constitutes but a small percentage of total nitrogen. When kidney function is normal, it is permissible to assume an average value of 0.03 gm. for non-protein nitrogen and to subtract this value from the total nitrogen value before obtaining the value for protein, and from the albumin nitrogen plus NPN similarly.

B. *Determination of Fibrinogen*.—1. *Reagents*.—In addition to the preparation of the other blood proteins, the

Add 5.36 cc. of concentrated sulfuric
100 cc.

(b) *Sodium Hydroxide Solution*, 1 per cent.

(c) *Calcium Chloride Solution*, 2.5 per cent, prepared from the anhydrous salt.

2. Procedure.—Collect about 5 cc. of blood in a flask or test tube containing 1.5 to 2 mg. of potassium or lithium oxalate per cc. of blood. Separate the plasma by centrifugalizing. Pipet 1 cc. of the plasma into a 50-cc. beaker containing 25 cc. of 0.9 per cent NaCl solution plus 1 cc. of 2.5 per cent CaCl_2 solution.

Swirl gently and let stand at least thirty minutes until a solid clot has formed. Insert a glass rod into the beaker and swirl gently, loosening the clot and winding it on to the glass rod. Rotate the glass rod bearing the clot between filter papers to dry. Place the rod and clot in a centrifuge tube, add 4 cc. of 1 per cent sodium hydroxide, and place in a boiling water bath for several minutes until the fibrin lump comes off the rod. Add 10 cc. of water, using part of it to wash the rod, which is removed from the centrifuge tube. Centrifugalize for five minutes at about 2000 to 3000 r.p.m., then transfer the supernatant liquid to a 25-cc. volumetric flask. To the precipitate in the centrifuge tube, add 5 cc. of water and mix with the precipitate by tapping the tube, then centrifugalize again. Decant the supernatant into the flask containing the first supernatant. Add 1 cc. of 5 per cent sulfuric acid and dilute to the 25-cc. mark with distilled water.

Transfer a 5 cc. aliquot of the solution to a pyrex digestion tube. Digest, nesslerize, and compare in a colorimeter in the same manner as for total protein. Filters should be used as given under Total Protein, page 222.

3. Calculation. $\frac{R_s}{R_u} \times 0.625$ equals grams of fibrinogen per 100 cc. of plasma.

XX. **Specific Gravity of Whole Blood and Plasma; Calculation of Plasma Proteins, Hemoglobin and Hematocrit** (Copper Sulfate Method of Phillips, Van Slyke *et al.**)

A method has been devised, using copper sulfate solutions of known, accurate specific gravity, by means of which the specific gravities of whole blood and plasma may be determined and from these gravities, by the use of line charts, the plasma proteins, hemoglobin and hematocrit calculated.

Small drops of whole blood or plasma are allowed to fall into a graded series of copper sulfate solutions of known specific gravities. Upon contact with the solution, each drop becomes encased in copper proteinate and remains as a discrete drop without change in specific gravity for fifteen to twenty seconds. Drop size need not be constant and no temperature corrections are necessary.

1. Reagents.—(a) *Heparin*, or

(b) *Oxalate Mixture* (Heller and Paul).—Dissolve 3 gm. ammonium oxalate and 2 gm. potassium oxalate in 250 cc. of water. Pipet into pyrex test tubes, 125 x 16 mm., 0.25 cc. of the oxalate solution. Spread in a film over the lower walls and dry in an incubator at not over 50° C.

(c) *Copper Sulfate*, crystalline ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). This should be pulverized or fine enough to pass through a No. 20 mesh.

(d) *Copper Sulfate Solution*.—A saturated solution used to prepare the stock solution of gravity 1.100 \pm 0.0003.

Place 4 pounds of finely pulverized copper sulfate in a 4-liter bottle

* U. S. Navy Research Unit, Hospital of the Rockefeller Institute for Medical Research, New York City.

and add 2500 cc. of water. Shake vigorously for a total of five minutes which need not be continuous. Take the temperature of the supernatant solution to the nearest half degree C. *immediately* at the end of the shaking period, *i. e.*, at the *moment saturation is completed*. Decant the supernatant *immediately* filtering through dry paper into a clean, dry 4-liter bottle. Undissolved crystals may be used again.

TABLE 21.—SATURATED COPPER SULFATE SOLUTION TO BE DILUTED TO 1 LITER TO MAKE THE STOCK SOLUTION OF SPECIFIC GRAVITY 1.100

(Temperature in °C. or °F. refers to the temperature of the saturated solution at the time of saturation)

Temperature			Temperature			Temperature		
°C.	°F.	Cc.	°C.	°F.	Cc.	°C.	°F.	Cc.
10 0	50.0	578	20 0	68 0	458	30 0	86 0	425
10 5	50 9	573	20 5	68 9	484	30 5	86 9	423
11 0	51 8	568	21 0	69 8	480	31.0	87.8	420
11 5	52 7	563	21 5	70 7	477	31 5	88.7	417
12 0	53 6	558	22 0	71 6	473	32 0	89.6	414
12 5	54 5	553	22 5	72 5	469	32 5	90 5	412
13 0	55 4	548	23 0	73 4	466	33 0	91 4	409
13 5	56 3	543	23 5	74 3	463	33 5	92 3	406
14 0	57 2	539	24 0	75 2	460	34 0	93.2	403
14 5	58 1	534	24 5	76 1	456	34 5	94 1	401
15 0	59 0	529	25 0	77 0	453	35 0	95 0	398
15 5	59 9	525	25 5	77 9	450	35 5	95 9	395
16 0	60 8	521	26 0	78 8	447	36 0	96 8	392
16 5	61 7	516	26 5	79 7	445	36 5	97 7	390
17 0	62 6	512	27 0	80 6	442	37 0	98 6	387
17 5	63 5	508	27 5	81 5	439	37 5	99 5	384
18 0	64 4	504	28 0	82 4	436	38 0	100 4	381
18 5	65 3	500	28 5	83 3	434	38 5	101 3	379
19 0	66 2	496	29 0	84 2	431	39 0	102 2	376
19 5	67 1	492	29 5	85 1	428	39 5	103 1	373
20 0	68 0	488	30 0	86 0	425	40 0	104 0	370

(e) *Copper Sulfate Solution, Gravity 1.100.*—Use the saturated solution *at once* to prepare the stock solution, sp. gr. 1.100. Measure the volume of saturated solution indicated in Table 21, in a 500-cc. cylinder and pour it into a liter volumetric flask. Drain the cylinder for thirty seconds. Fill the flask to the mark with distilled water and mix. A contraction results

each time and discarding the rinsings. The stock solution and the standards described below should all be prepared at a temperature within 5° C. of that at which the saturated solution was made.

Once prepared, the standards may be used at any temperature within $\pm 15^\circ$ C. of that at which prepared.

(f) *Standard Solutions, in 100-cc. portions.*—Using a buret filled with stock solution, sp. gr. 1.100, run into a 100-cc. volumetric flask the amount indicated in Table 22. Dilute to the mark, mix and transfer to a 4-ounce "oval prescription bottle." Stopper at once. Rinse the flask, discarding the rinsings and prepare the next standard.

Continue until the entire set from sp. gr. 1.008 to 1.075 is prepared. This set covers the extreme ranges for blood plasma, ascitic fluid and transudates. Each standard is correct to within 0.0001.

TABLE 22.—CC. OF STOCK COPPER SULFATE SOLUTION OF GRAVITY 1.1000 TO BE DILUTED TO 100 CC., 50 CC. OR 25 CC. TO PREPARE STANDARD SOLUTIONS OF GRAVITY, G, TO WITHIN ± 0.0001

G	100	50	25	G	100	50	25
1 008	7 33	3 67	1 84	41	40 00	20 00	10 00
9	8.32	4 16	2 08	42	41 00	20 50	10 25
10	9 31	4 66	2 33	43	42 00	21 00	10 50
				44	43 00	21 50	10 75
11	10 30	5 15	2 58	45	44 00	22 00	11 00
12	11 29	5 65	2 83				
13	12 28	6 14	3 07	46	45 00	22 50	11 25
14	13 27	6 64	3 32	47	46 00	23 00	11 50
15	14 26	7 13	3 57	48	47 00	23 50	11 75
				49	48 00	24 00	12 00
16	15 25	7 63	3 82	50	49 00	24 50	12 25
17	16 24	8 12	4 06				
18	17 23	8 62	4 31	51	50 00	25 00	12 50
19	18 22	9 11	4 56	52	51 00	25 50	12 75
20	19 21	9 61	4 81	53	52 00	26 00	13 00
				54	53 00	26 50	13 25
21	20 20	10 10	5 05	55	54 00	27 00	13 50
22	21 19	10 60	5 30				
23	22 17	11 09	5 56	56	55 00	27 50	13 75
24	23 15	11 58	5 79	57	56 00	28 00	14 00
25	24 14	12 07	6 04	58	57 00	28 50	14 25
				59	58 00	29 00	14 50
26	25 12	12 55	6 28	60	59 00	29 50	14 75
27	26 10	13 05	6 53				
28	27 08	13 54	6 77	61	60 00	30 00	15 00
29	28 06	14 03	7 02	62	61 00	30 50	15 25
30	29 04	14 52	7 26	63	62 00	31 00	15 50
				64	63 00	31 50	15 75
31	30 00	15 01	7 51	65	64 00	32 00	16 00
32	31 00	15 50	7 75	66	65 00	32 50	16 25
33	32 00	16 00	8 00	67	66 00	33 00	16 50
34	33 00	16 50	8 25	68	67 00	33 52	16 76
35	34 00	17 00	8 50	69	68 10	34 04	17 02
				70	69 10	34 56	17 28
36	35 00	17 50	8 75	71	70 20	35 08	17 54
37	36 00	18 00	9 00	72	71 20	35 60	17 80
38	37 00	18 50	9 25	73	72 20	36 12	18 06
39	38 00	19 00	9 50	74	73 30	36 64	18 32
40	39 00	19 50	9 75	75	74 30	37 15	18 58

G = Specific gravity of standard solution

100 = Cc of 1.100 stock solution diluted to 100 cc

50 = Cc of 1.100 stock solution diluted to 50 cc.

25 = Cc. of 1.100 stock solution diluted to 25 cc.

Standards may be prepared, accurate to 0.0003 by a somewhat simpler method. To make the standard of 1.075 gravity, measure 74 cc. of stock from a buret into a 100-cc volumetric flask. Dilute to the mark, mix and transfer to a 4-ounce bottle which is stoppered to prevent evaporation. For the next standard, 1.074, in the series, after rinsing the flask take 73 cc of stock and dilute to the mark.

For each subsequent standard take an amount of stock which is 1 cc less than the desired specific gravity. By doing this allowance is made for the shrinkage in volume which occurs on mixing.

A complete or partial set may be prepared. Sets of standards of 50 cc. or 25 cc. each may be prepared if the amount of work to be done would so indicate. Any standard may be used until about one-fortieth of its volume of blood or plasma has been added before the gravity is changed by 0.0005. One small drop for each cc. of standard is approximately that amount. The 100-cc. standards, therefore, serve for about 100 tests.

Controls to determine when standards should be changed may be prepared by adding 2.5 cc. of plasma to a standard of 1.028 gravity (100 cc. in a 4-ounce bottle) and 2.5 cc. of whole blood to a standard of 1.060 gravity. When the volume of precipitate in a standard equals that in the control bottle, the standard should be replaced.

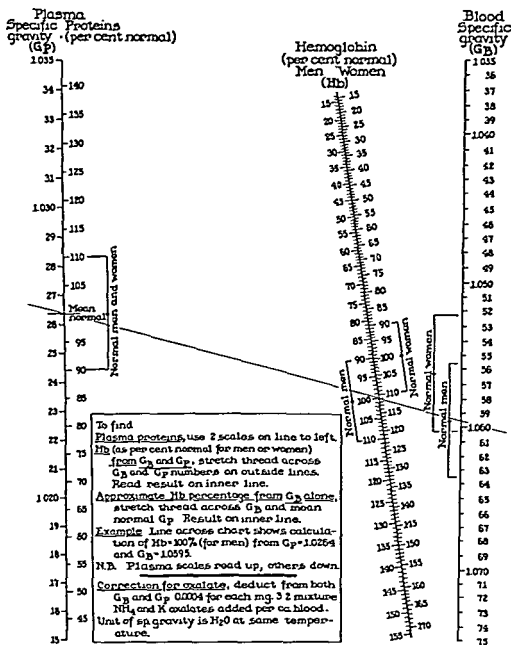


FIG. 22.—Line chart for calculating plasma proteins, hemoglobin and hematocrit from gravities of plasma and blood. (U. S. Navy Research Unit, Hospital of the Rockefeller Institute for Medical Research, New York City.)

2. Procedure.—In drawing blood by venepuncture do not allow the tourniquet to remain on for more than one minute. Drops of whole blood may be delivered directly into the standards from the syringe and needle.

If plasma is to be used as well, discharge the blood into an oxalate tube and mix thoroughly. Centrifugalize a portion to obtain clear plasma. Mix cells and plasma very thoroughly by inverting at least ten times if oxalated whole blood is used. Serum may be used instead of plasma if the correction given under "Calculations" is applied.

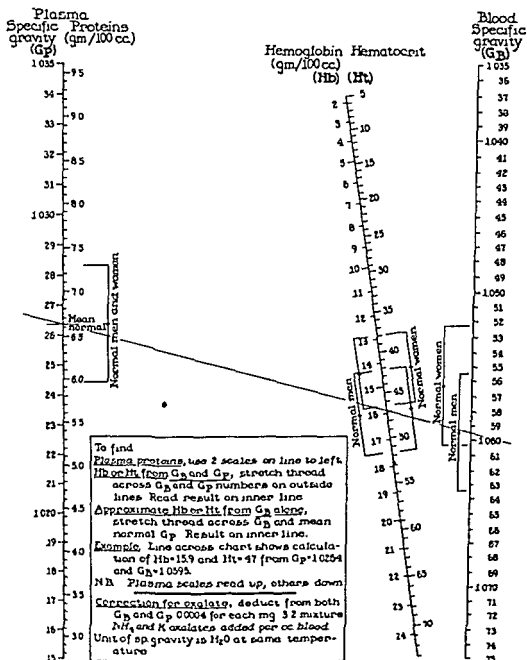


FIG. 23 — Line chart for calculating percentages of normal plasma proteins and hemoglobin from gravities of plasma and blood. (U. S. Navy Research Unit, Hospital of the Rockefeller Institute for Medical Research, New York City)

The drop of plasma, whole blood or serum is delivered from a medicine dropper or the syringe needle from a height of about 1 cm. above the solution. Small drops should be used since they permit more tests before

a standard must be changed. Greasing the tip of the medicine dropper with vaseline mixed with caprylic alcohol will reduce the drop size.

Within five seconds the momentum of the fall is lost and the drop will rise, remain stationary or continue to fall. The gravity of the drop does not change for an additional ten to fifteen seconds and there is ample time to observe its behavior during this time. If the drop is lighter than the solution it will rise, perhaps only a few millimeters and may then sink again. If the drop is heavier it will continue to fall. If of the same gravity it will remain stationary for ten to fifteen seconds and then fall. The action of the drop during the ten seconds after it has lost its momentum determines whether it is lighter or heavier than, or the same as the test solution.

3. Calculations.—Whole blood gravities G_b and plasma gravities G_p as determined are used to calculate plasma proteins, hemoglobin and hematocrit directly from the line charts, Figures 22 and 23.

If heparin is used as the anticoagulant no corrections are necessary.

If the oxalate mixture is used, subtract 0.0004 from the observed gravities for each mg. of oxalate mixture added per cc. of blood.

If serum is used instead of plasma, add 0.0005 to the observed gravity to get the plasma gravity.

XXI. Determination of Carbon Monoxide.

A. Carbon Monoxide of Blood (Sayers, Yant and Jones).—This method depends upon the fact that carbon monoxide, CO, combines with the hemoglobin of the blood, displacing the oxygen and changing oxyhemoglobin, HbO , to carbon monoxide hemoglobin, $HbCO$. The blood under examination is treated with absolutely fresh pyrotannic acid solution, which in the presence of CO develops a specific color. This color is compared with a set of standards representing various degrees of saturation with CO from 0 per cent to 100 per cent in steps of 10.

1. Reagents.—(a) *Pyrogallol*, 2 per cent aqueous solution.

(b) *Tannic Acid*, 2 per cent aqueous solution.—Equal volumes of (a) and (b) are mixed just prior to use.

(c) *Preparation of Standards.*—Five cubic centimeters or more of human or animal blood are drawn and kept from clotting by the addition of 5 mg. of sodium citrate per cc. of blood. This blood is divided into equal parts, one of which is immediately diluted 1 to 10 with distilled water, forming other is saturated with it. Where illuminating sulfuric acid with oxalic acid, passing the gas produced through a solution of sodium hydroxide to remove the carbon dioxide, before passing it through the blood. The saturated blood is now diluted 1 to 10 with distilled water, forming Solution II, all carbon monoxide hemoglobin.

From Solution I and Solution II, mixtures are made in small test tubes, approximately 8 mm. in diameter, which total 1 cc. but vary from 0 per cent to 100 per cent $HbCO$ in steps of 10. For example: To the first tube 1 cc. of the oxyhemoglobin, Solution I only, is added; to the second tube 0.9 cc. of Solution I and 0.1 cc. of Solution II; to the third tube 0.8 cc. of Solution I and 0.2 cc. of Solution II and so on in each of the succeeding tubes, Solution I diminishing by 0.1 cc. in each tube, and Solution II

increasing by 0.1 cc. in each tube until the last tube which contains 1 cc. of Solution II only and will represent 100 per cent HbCO.

To each standard thus prepared add 1 cc. of the mixture of equal parts of strictly fresh solutions of 2 per cent pyrogallol and 2 per cent tannic acid, after which the tube is inverted twice to insure thorough mixing. The tube should be sealed immediately by pouring a little melted paraffin on top of the contents while immersed in cold water to prevent overheating. When the walls of the tube become dry the remainder of the tube should be filled with ordinary sealing wax. Care should be taken to exclude all air. These standards develop their full color in about thirty minutes, and if kept in a cool, dark place will keep for one or two weeks.

2. Procedure.—Collect 0.1 cc. of blood from the tip of the finger and dilute with 0.9 cc. of distilled water in a test tube of the same size used in the preparation of the standards. Add 1 cc. of the strictly fresh pyrotannic acid mixture used in the preparation of the standards. Invert the tube several times. Place in a rack and allow to stand for fifteen minutes, at the end of which period comparison is made with the standards by interposing between them until the standard is found which it most nearly matches. If CO is indicated the tube should be allowed to stand for fifteen minutes longer and another reading made.

3. Calculation.—The percentage of HbCO is estimated from the value of the standard most closely matched.

B. Carbon Monoxide in Air (Sayers, Yant and Jones).—1. Reagents.—Same as for blood.

2. Procedure.—Fresh human or animal blood is obtained and diluted 1 to 10 with distilled water. Two cc. of this solution are then introduced into a sample bottle containing the air to be analyzed, the manipulation being such as to allow as little of the air as possible to escape.

The sample bottle is a 250-cc. bottle or flask in which the air to be analyzed is drawn through a glass tube on the end of a scrubber and bottle containing the blood solution. After the last squeeze of the bulb, the glass tube is quickly removed and a rubber stopper inserted in the bottle.

The bottle containing the blood is equilibrated by being rotated constantly for fifteen to twenty minutes, as much as possible of the surface of the bottle being covered with the blood solution. When this equilibration has been finished, 1 cc. of the equilibrated blood solution is placed in a test tube, 1 cc. of fresh pyrotannic acid mixture added, and the comparison of colors and determination of HbCO made according to the procedure for blood.

3. Calculation.
$$\frac{\text{HbCO}}{100 - \text{HbCO}} \times \frac{2093}{300} = \text{parts of CO per 10,000 parts of air}$$

Example.—A blood solution equilibrated with a gas sample was found to contain 50 per cent HbCO by comparison with the standards. Substituting this value in the above equation gives the following:

$$\frac{50}{100 - 50} \times \frac{2093}{300} = 7 \text{ parts CO per 10,000 parts of air}$$

The equilibration of the blood with the air sample should be done at a temperature not varying more than 3° C. plus or minus from 20° C. (5° plus or minus from 68° F.). Within this range the temperature correction is so small as to be negligible.

XXII. Alkali Reserve. Direct Method. Carbon Dioxide Capacity of the Blood Plasma by the Volumetric Method (Van Slyke and Cullen).

The plasma from oxalated blood is shaken in a separatory funnel filled with an air mixture whose carbon dioxide tension approximates that of normal arterial blood, by which treatment it combines with as much carbon dioxide as it is able to hold under normal tension. A known quantity of the saturated plasma is then acidified within a suitable pipet, and its carbon dioxide is liberated by the production of a partial vacuum. The liberated carbon dioxide is then placed under atmospheric pressure, its volume accurately measured, and the volume corresponding to 100 cc. of plasma calculated.

1. Reagents and Apparatus. — (a) *Apparatus.*—The apparatus used is illustrated in Figure 24. It is made of strong glass in order to withstand the weight of the mercury without danger of breaking, and is held in a strong clamp, the jaws of which are lined with rubber. In order to prevent accidental slipping of the apparatus from the clamp, an iron rod of 6 or 8 mm. diameter should be so arranged as to project under the cock "f" between "c" and "d." Three hooks or rings at the levels 1, 2 and 3 serve to hold the levelling bulb at different stages of the analysis. The bulb is connected with the bottom of the apparatus by a heavy-walled rubber tube.

It is necessary, of course, that both stopcocks be properly greased and absolutely air-tight; and it is also essential that they (especially "f") should be held in place so that they cannot be forced out by pressure of the mercury. Rubber bands may be used for this purpose, but it has been found that elastic fine wire

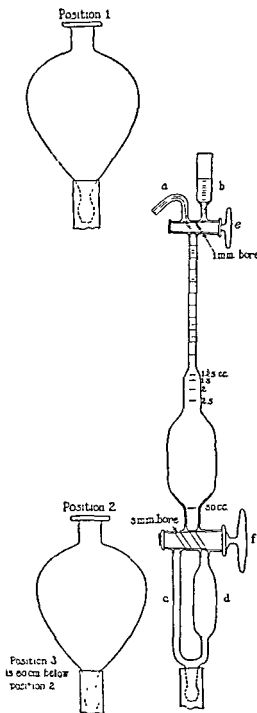


FIG. 24 —Van Slyke volumetric CO_2 apparatus.

spirals, applied in the same manner as the rubber bands, are stronger and more durable.

When the apparatus is new, or whenever air has been admitted to it

as in renewing mercury or, perhaps, through leaks, the rubber tubing and even the glass walls, may contain considerable amounts of gases; these must be evacuated before using it. To test the apparatus for tightness and freedom from gases, raise the levelling bulb and completely fill the apparatus with mercury. The bulb is then lowered to Position 3, so that a vacuum is produced, the mercury falling to about the middle of "d;" the bulb is then raised again. If the apparatus is tight and gas-free, the mercury will refill it completely, and will strike the upper cock with a sharp click. If there is any gas in the apparatus, it will act as a cushion; the click will not be heard, and a bubble will remain above the mercury. In this case expel the collected gas through cock "e," and repeat the procedure until all the gas has been evacuated. After the apparatus has once been freed from gases, it can be used repeatedly and indefinitely without further trouble from this source, if no air is admitted and there is no leak. It is always desirable however, before making the first determination of a series, to test the apparatus as described above.

After a determination is finished, the levelling bulb is lowered without opening the upper cock and most of the mercury is withdrawn from the pipet through "c." The water solution from "d" is readmitted; and, with levelling bulb raised to Position 1, the water solution together with a little mercury is forced out of the apparatus through "a." The apparatus may then be used immediately for another determination; the slight amount of the old solution which remains on the inner surface of the pipet is negligible; if it is to be set aside it is well to rinse it several times with water, and then to leave it completely filled with mercury. If the apparatus is used only occasionally, it is well to stopper lightly both cup "b" and the mercury bulb.

(b) *Sulfuric Acid*, 5 per cent.

(c) *Octyl Alcohol*.

2. Procedure.—(a) *Drawing the Blood*.—About 6 to 7 cc. of venous blood are drawn into a centrifuge tube containing a very little powdered potassium oxalate. Use 20 mg. for 10 cc. of blood, and not more; less should be used if less blood is drawn. The estimation may be made with as little as 3 cc. of blood, using only 0.5 cc. of plasma; but the larger quantity of blood, allowing 1 cc. of plasma to be used, is preferable. The addition to the oxalate in the tube, before the blood is drawn, of sufficient paraffin oil to cover the quantity of blood taken is also recommended but if the determination is proceeded with promptly, and if only the very minimum of shaking or stirring sufficient to mix the blood and oxalate is done, the paraffin oil can be omitted.

The tube and contents are then centrifugalized to obtain the separated plasma.

If it is necessary to make the estimation of carbon dioxide at a later time, the plasma should be transferred to a paraffined tube, covered with paraffin oil, stoppered lightly and kept cold. Under these conditions, if sterile, it may be kept for over a week without alteration of its carbon dioxide capacity.

(b) *Saturation of the Plasma with Carbon Dioxide*.—Transfer about 3 cc. of the plasma to a 300-cc. separatory funnel; the air within the funnel is displaced either by alveolar air from the lungs of the operator or 5.5 per cent carbon dioxide mixture from a tank. In either case the gas mixture

must be passed over moist glass beads before it enters the funnel; this may be done conveniently by the use of some such apparatus as is shown in Figure 25.

When alveolar air is used, the operator, without inspiring more deeply than normal, expires as quickly and as completely as possible through the glass beads and separatory funnel. The stopper of the funnel should be inserted just before the expiration is finished, so that no air will be drawn back into the funnel; the funnel-cock is then closed. In order to saturate the plasma, the separatory funnel is slowly turned for two minutes, distributing the plasma in a thin layer as completely as possible over the interior surface of the funnel.

After saturation is complete, the stoppered funnel is placed upright and allowed to stand until the fluid has drained from the walls into the bottom.

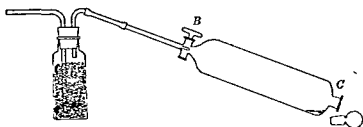


FIG. 25.—Separatory funnel used in saturating blood plasma with carbon dioxide.

(c) *Determination of the Carbon Dioxide.*—Before beginning a determination, it is necessary that the entire apparatus, including the chamber "d," the communicating tube "c," and the pipet proper, be filled with mercury to the top of both capillary tubes above the cock "e;" this is done by raising the mercury bulb with the cock open, and closing the cock when the mercury reaches the proper level. Then, using a cotton-covered applicator, wash out the cup "b" with distilled water and remove any excess with a dry applicator. This washing of the cup is essential to prevent any premature liberation of carbon dioxide from the plasma, especially since the last solution added in any preceding determination was the 5 per cent sulfuric acid.

Then, the tight and gas-free apparatus being filled with mercury, 1 cc. of plasma, accurately pipeted, is placed in the cup, the tip of the pipet remaining below the surface of the plasma as it is added. With the mercury bulb at Position 2, the plasma is admitted from the cup into the 50-cc. chamber, leaving just enough above the cock "e" to fill the capillary, so that no air will be introduced when the next solution is added. The cup is then washed with two portions of about 0.5 cc. of water, each portion being admitted to the pipet in turn. A small drop of octyl alcohol is then permitted to flow entirely into the capillary above "e" and, finally, 0.5 cc. of 5 per cent sulfuric acid are run in.

It is not necessary that exactly 1 cc. of wash water and 0.5 cc. of acid shall be taken but the total volume of the water solution introduced must extend exactly to the 2.5-cc. mark on the apparatus; this can readily be accomplished by placing slightly more than sufficient acid solution in the cup, and then drawing the solution down exactly to the mark.

After the acid has been added, the cock "e" is closed, a drop of mercury is placed in the cup and allowed to run down the capillary as far as the cock, in order to seal the latter. Whatever excess of sulfuric acid remains is then removed by a cotton-covered applicator.

If the amount of plasma available is small, saturate a little more than 0.5 cc. with carbon dioxide in a 50-cc. funnel, and use exactly 0.5 cc. for the determination. In this case the quantities of water and acid used to wash the plasma into the apparatus are halved, so that the total volume of water solution introduced is only 1.25 cc. and the observed volume of gas is multiplied by 2 before proceeding with the calculation.

The mercury bulb is now lowered and hung at Position 3, and the mercury in the pipet allowed to run down to the 50-cc. mark, producing a vacuum in the apparatus. When the mercury meniscus has fallen to the 50-cc. mark, the lower cock "f" is closed, and the pipet removed from the stand. Equilibrium of the carbon dioxide between the 2.5 cc. of water solution and the 47.5 cc. of free space is obtained by turning the pipet upside down 15 or more times, thus thoroughly agitating the contents. The pipet is then replaced in the clamp.

By opening the cock "f," the water solution is now allowed to flow from the pipet completely into the chamber "d," without, however, allowing any c
the left hand,
the pipet with
evelling bulb is then raised in
"f" is turned so as to connect
from "c" fills the body of the
pipet and as much of the calibrated stem at the top as is not occupied by the gas extracted from the solution. A few hundredths of a cc. of water, which could not be completely drained into "d," floats on top of the mercury in the pipet; the error caused by the reabsorption of carbon dioxide into this small volume of water is negligible if the reading is made at once. The mercury bulb is held at such a level that the gas in the pipet is under atmospheric pressure; that is, level with the top of the mercury column in the pipet stem, and the volume of gas read on the scale.

In order to have the column in the pipet exactly balanced by that outside, the surface of the mercury in the levelling bulb should be raised until it is level with the mercury meniscus in the pipet, and then, for entire accuracy, raised above the latter meniscus by a distance equal to one-thirteenth of the height of the column of water above the mercury. As the water column should never be more than 10 mm. high, the correction that has to be estimated is less than 1 mm. of mercury, not enough to influence results appreciably.

3. *Calculation.*—When from plasma, saturated as above described with alveolar air, gases are extracted for analysis, one obtains, not only the carbon dioxide bound as bicarbonate and set free by acidification, but also the carbon dioxide and air physically dissolved by the plasma and water. The gases thus dissolved are, of course, independent of the alkali reserve, and are subtracted from the total in order that the carbon dioxide bound as bicarbonate may be estimated. The exact amount to be subtracted varies slightly with room temperature, and may be determined by blank analysis, or calculated from the known solubility coefficients of the gases. This has been done; and all of the necessary corrections are included in Table 23. This table has been so calculated as to give directly, for any given temperature and barometric pressure, the volume of carbon dioxide,

reduced to 0° C. and 760 mm. of mercury pressure, that 100 cc. of plasma are capable of binding when saturated at 20° C. with carbon dioxide at approximately 41 mm. tension.

TABLE 23—CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA. (VAN SLIKE AND CULLEN.)

Observed vol gas B × $\frac{760}{\text{B}}$	Cc. of CO ₂ reduced to 0° C., 760 mm., bound as bicarbon- ate by 100 cc of plasma				Observed vol. gas B × $\frac{760}{\text{B}}$	Cc. of CO ₂ reduced to 0° C., 760 mm., bound as bicarbon- ate by 100 cc of plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0 20	9 1	9 9	10 7	11 8	0 60	47 7	48 1	48 5	48 6
1	10 1	10 9	11 7	12 6	1	48 7	49 0	49 4	49 5
2	11 0	11 8	12 6	13 5	2	49 7	50 0	50 4	50 4
3	12 0	12 8	13 6	14 3	3	50 7	51 0	51 3	51 4
4	13 0	13 7	14 5	15 2	4	51 6	51 9	52 2	52 3
5	13 9	14 7	15 5	16 1	5	52 6	52 8	53 2	53 2
6	14 9	15 7	16 4	17 0	6	53 6	53 8	54 1	54 1
7	15 9	16 6	17 4	18 0	7	54 5	54 8	55 1	55 1
8	16 8	17 6	18 3	18 9	8	55 5	55 7	56 0	56 0
9	17 8	18 5	19 2	19 8	9	56 5	56 7	57 0	56 9
0 30	18 8	19 5	20 2	20 8	0 70	57 4	57 6	57 9	57 9
1	19 7	20 4	21 1	21 7	1	58 4	58 6	58 9	58 8
2	20 7	21 4	22 1	22 6	2	59 4	59 5	59 8	59 7
3	21 7	22 3	23 0	23 5	3	60 3	60 5	60 7	60 6
4	22 6	23 3	24 0	24 5	4	61 3	61 4	61 7	61 6
5	23 6	24 2	24 9	25 4	5	62 3	62 4	62 6	62 5
6	24 6	25 2	25 8	26 3	6	63 2	63 3	63 6	63 4
7	25 5	26 2	26 8	27 3	7	64 2	64 3	64 5	64 3
8	26 5	27 1	27 7	28 2	8	65 2	65 3	65 5	65 3
9	27 5	28 1	28 7	29 1	9	66 1	66 2	66 4	66 2
0 40	28 4	29 0	29 6	30 0	0 80	67 1	67 2	67 3	67 1
1	29 4	30 0	30 5	31 0	1	68 1	68 1	68 3	68 0
2	30 3	30 9	31 5	31 9	2	69 0	69 1	69 2	69 0
3	31 3	31 9	32 4	32 8	3	70 0	70 0	70 2	69 9
4	32 3	32 8	33 4	33 8	4	71 0	71 0	71 1	70 8
5	33 2	33 8	34 3	34 7	5	71 9	72 0	72 1	71 8
6	34 2	34 7	35 3	35 6	6	72 9	72 9	73 0	72 7
7	35 2	35 7	36 2	36 5	7	73 9	73 9	74 0	73 6
8	36 1	36 6	37 2	37 4	8	74 8	74 8	74 9	74 5
9	37 1	37 6	38 1	38 4	9	75 8	75 8	75 8	75 4
0 50	38 1	38 5	39 0	39 3	0 90	76 8	76 7	76 8	76 4
1	39 1	39 5	40 0	40 3	1	77 8	77 7	77 7	77 3
2	40 0	40 4	40 9	41 2	2	78 7	78 6	78 7	78 2
3	41 0	41 4	41 9	42 1	3	79 7	79 6	79 6	79 2
4	42 0	42 4	42 8	43 0	4	80 7	80 5	80 6	80 1
5	42 9	43 3	43 8	43 9	5	81 6	81 5	81 5	81 0
6	43 9	44 3	44 7	44 9	6	82 6	82 5	82 4	82 0
7	44 9	45 3	45 7	45 8	7	83 6	83 4	83 4	82 9
8	45 8	46 2	46 6	46 7	8	84 5	84 4	84 3	83 8
9	46 8	47 1	47 5	47 6	9	85 5	85 3	85 2	84 8
0 60	47 7	48 1	48 5	48 6	1 00	86 5	86 2	86 2	85 7

In compiling this table, it is assumed that the saturation of the plasma with carbon dioxide, and the determination of the amount of gas, are both done at the same temperature and pressure; and the second step should, therefore, immediately follow the first.

The temperature figures at the heads of the columns represent room temperature at which the procedure is carried out; and "B" represents the observed barometric pressure.

To make the calculations, after determining the volume of carbon dioxide in the plasma, divide the observed pressure by 760, and multiply the result by the volume of carbon dioxide as read on the apparatus.

Then, opposite this figure in the left hand column of the table, read the desired corrected volume of carbon dioxide under the temperature heading nearest to the observed temperature.

This gives directly the cc. of carbon dioxide, reduced to 0° C., and 760 mm. of mercury pressure, bound as bicarbonate by 100 cc. of plasma; or, in other words, the combining power of the plasma expressed directly in "volumes per cent."

XXIII. Determination of Atabrine* in Blood and Urine (Masen†).—This method and that of Brodie and Udenfriend which follows are based upon the extraction of atabrine with an immiscible solvent and its subsequent estimation by measuring its fluorescence as compared with that of a standard in a photoelectric fluorometer.

Masen extracts the atabrine from whole blood or plasma after alkalization with strong sodium hydroxide, by means of a mixture of petroleum ether, isopropyl and isobutyl alcohols. The solvent mixture plus the atabrine is purified by washing with dilute NaOH and the atabrine then returned to the aqueous phase by shaking with dilute HCl. After making this solution alkaline, the fluorescence which is increased thereby, is measured.

With plasma or urine comparison of fluorescence is made against a simple aqueous standard atabrine solution. However, in the case of whole blood recovery of atabrine has been found to approximate only 95 to 97 per cent of the total present. It is therefore necessary to use a standard prepared by adding atabrine solution to whole blood and then treating it in the same manner as the unknowns. Since the losses are comparable in standard and unknown no correction for incomplete recovery is necessary.

1. **Reagents and Apparatus.**—(a) *Sodium Hydroxide*, 4 N.

(b) *Sodium Hydroxide*, 0.3 N.

(c) *Petroleum Ether*, b. p. 30° to 65° C., redistilled.

(d) *Isopropyl-isobutyl Alcohol Mixture*.—Mix equal parts of redistilled isopropyl and isobutyl alcohols.

(e) *Isopropyl Alcohol in 0.1 N HCl*.—Measure 300 cc. of redistilled isopropyl alcohol into a liter volumetric flask, add 100 cc. of 1 N HCl and dilute to the mark with distilled water.

(f) *Borate-NaOH Buffer*.—Dissolve 4 gm. of sodium borate in 100 cc. of 1.35 N sodium hydroxide. Filter until clear.

(g) *Standard Atabrine Solutions*.—(1) *Stock solution*. Dissolve 63.6 mg. of atabrine hydrochloride ($C_{23}H_{30}ClN_3O \cdot 2HCl \cdot 2H_2O$) in a liter of 0.1 N hydrochloric acid. In a dark bottle in the refrigerator this solution will keep for one month. (2) *Working Standard*. Dilute 3 cc. of stock solution to 100 cc. with 0.1 N HCl. Each cc. contains 0.0015 mg. of atabrine base ($C_{23}H_{30}ClN_3O$). This dilute solution deteriorates rapidly and must be prepared on the day of use.

(h) *Photoelectric Fluorometer*.—Any instrument having sufficient sensitivity for the determination of thiamine or riboflavin in biological material is satisfactory. The Coleman Electronic Photofluorometer No. 12, shown

* Quinacrine hydrochloride is the name made official by the U S P. XII.

† MASEN, J. M.: J. Biol. Chem., 148, 529, 1943.

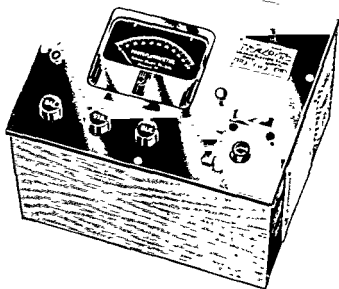


FIG. 26.—Electronic photofluorometer, Model 12. (Courtesy of the Coleman Electric Co.)

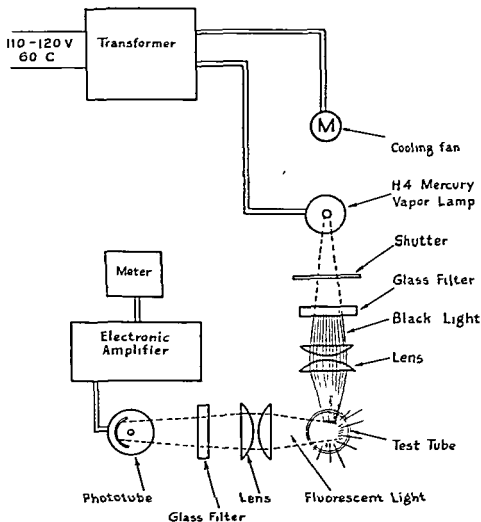


FIG. 27 —Illustrative diagram of Coleman Model 12 photofluorometer. (Courtesy of the Coleman Electric Co.)

in Figures 26 and 27, is very satisfactory. It combines ruggedness and stability with sensitivity and simplicity of operation.

The source of activating light in the photofluorometer is an ultra-violet lamp. This is provided with a shutter so that light may be excluded from the system except during the actual reading of fluorescence. When the shutter is opened light from the lamp passes through a filter which transmits only a narrow band in the range 4200\AA° . This band of light strikes the atabrine (or any other solution) in the fluorometer cuvet or tube and activates the atabrine to fluorescence. The fluorescent light passes through a second filter and strikes a photoelectric cell. This second filter transmits practically none of the incident light since the paths of the incident and emitted light are at right angles to each other and the filters are complementary.

The current generated by the photocell is, therefore, almost entirely due to the emitted fluorescent light and the galvanometer deflection produced by it after amplification is directly proportional to the intensity of fluorescence.

Since the fluorescence of atabrine (and many other compounds) in dilute solution is directly proportional to concentration, unknowns can be directly determined by comparison with known standards.

(i) *Filters*.—Between the ultra-violet lamp and the sample use Corning blue-glass filter No. 511, thickness 2 mm. Between sample and photocell use Corning glass filter No. 338, 2 mm. thick. A metal screen intensity reducer (Coleman) is also required.

(j) *Stopcock Lubricant, Non-fluorescent*.—Heat 3 parts of glycerol and 2 parts of soluble starch to 140°C . until a smooth mixture results.

(k) *Extraction Vessels*.—Small cylindrical separatory funnels of about 30 cc. capacity such as are used in the determination of thiamine. These reaction vessels fit in the regular 50-cc. trunnion cups of the ordinary centrifuge. They are illustrated in Figure 28.

2. *Procedure*.—(a) *For Whole Blood*.—Collect blood by venepuncture using 1.5 mg. of lithium oxalate or 2 mg. of potassium oxalate per cc. as an anti-coagulant. If only one determination is to be made, take 12 cc. of blood. If several are to be made, collect 12 cc. from one patient and 6 cc. from the others. Prepare the extraction vessels, one for each sample, by placing in each 3 cc. of 1 N NaOH and 8 cc. of isopropyl-isobutyl alcohol mixture. Then add 1 cc. of water and 7 cc. of petroleum ether. In one of the vessels put 1 cc. of standard atabrine solution instead of the 1 cc. of water. This is to serve as the standard upon which to base the calculations.

To each vessel add 5 cc. of blood, using the additional 5 cc. from the 12 cc. specimen for the standard vessel. Stopper each as soon as the blood has been added and mix by inverting several times. Shake vigorously by hand or in a mechanical shaker for five minutes.

Allow the vessels to stand for three minutes for separation of the layers to occur, then draw off the blood layer as completely as possible and discard it. Whirl the tube to loosen protein particles clinging to the walls and centrifugalize at 1000 r.p.m. for three minutes. Draw off the small additional amount of blood which collects.

Add 10 cc. of 0.3 N NaOH to each vessel, stopper and shake vigorously for thirty seconds. After stratification is complete (one to two minutes) draw off and discard the lower alkaline layer. Centrifugalize for three minutes at 1000 r.p.m., then discard the small aqueous alkaline layer.

Next add 10 cc. of the 30 per cent isopropyl alcohol solution in 0.1 N HCl, stopper and shake vigorously for thirty seconds. The atabrine passes from the upper ether-alcohol layer to the lower aqueous-acid-alcohol layer. Centrifugalize for five minutes at 1000 r.p.m. to secure better separation of layers.

Draw off an aliquot of this lower layer allowing the first few drops to wash out the stem of the funnel. The amount withdrawn will depend upon how much is required to fill the fluorometer cuvetts to a point which will give a satisfactory reading. For most tubes 8 cc. are a proper aliquot. The tubes or cuvetts should be marked at the proper level. If a larger amount of fluid is required for the reading, use a greater volume of the aqueous-acid-alcohol solution in the extraction.

Prepare a blank by placing 8 cc. of isopropyl alcohol-acid solution in another cuvet. To each add 1 cc. of the NaOH-borate buffer and mix by vigorous shaking. Measure the fluorescence immediately after adding the alkali.

(b) *Procedure for Plasma*.—The technic is the same as that given for whole blood, using 5 cc. of plasma. Separation of plasma from cells must be carefully done in order to avoid including leucocytes in which the concentration of atabrine is many times greater.

The standard for comparison with plasma consists of 1 cc. of the working atabrine standard plus 4 cc. of water.

(c) *Procedure for Urine*.—Since the concentration of atabrine in the

of this dilution with 1 cc. of the NaOH-borate buffer and read in the fluorometer, using an appropriate aqueous solution of atabrine as the standard.

(d) *Measurement of Fluorescence*.—With the filters in place turn on the fluorometer ten minutes before the measurements are to be made so that

until the galvanometer reads some convenient figure, usually 75 to 100. The unknowns are then read as rapidly as possible, checking after each one or two with the standard solution to see that there has been no change from the initial setting. Should such change occur, reset the instrument with the sensitivity control and recheck the unknown.

Should a high value be encountered, set the standard to a lower value with the sensitivity control. If it is impossible to keep the galvanometer needle on the scale by this means, insert the metal screen intensity reducer in the filter holder, adjust the sensitivity with the standard in place and then read the unknown.

3. *Calculation*.—The readings of the fluorometer are directly proportional to the atabrine concentrations within the limits usually encountered.

$$\frac{R_u \times C_s \times 200}{R_s} = \text{mg. of atabrine per liter, where}$$

R_u = reading of unknown blood sample

R_s = reading of blood sample to which atabrine was added minus the reading of the same blood without the added atabrine, and

C_s = amount of atabrine in mg. added to the blood used as a standard

The following example illustrates typical results: Reading of blood to which 1 cc. of working atabrine standard solution was added is 75; reading of the same blood without added atabrine is 25. The difference, 75-25, or 50 scale divisions, is equal to the amount of atabrine added, which was 0.0015 mg. (1.5 gamma). Since 5 cc. of blood were used the factor 200 is used to bring the results to those per liter. Substituting the numerical values

$$\frac{25 \times 0.0015 \times 200}{50} = 0.15 \text{ mg per liter}$$

XXIV. Estimation of Atabrine in Plasma (Brodie and Udenfriend*).—Brodie and Udenfriend postulate that the antimalarial activity of atabrine is a simple function of its concentration in plasma and have devised their method so as to make only this measurement.

They have found the concentration in leucocytes to be of the order of 400 times that in plasma and that, therefore, changes in atabrine concentration in whole blood are as likely to reflect changes in leucocyte count during therapy as they are actual concentrations of the drug.

The atabrine is extracted from the plasma with ethylene dichloride at pH 8. It is then returned as a salt to an aqueous phase of 85 per cent lactic acid. Difficulty has been experienced because of the fluorescence of this material increases the difficulty of use. Violet light has also been used.

1. Reagents and Apparatus.—(a) *Stock Standard Atabrine Solution.*—Dissolve 119 mg. of atabrine dihydrochloride in about 200 cc. of water and dilute to 1 liter. This solution contains 0.1 mg. (100 gamma) of atabrine and should be kept in a dark bottle in the refrigerator, the

1 cc. of stock standard to 100 cc. solution should be prepared each day.

(b) *Ethylene Dichloride.*—Small amounts of fluorescent foreign material do not interfere as a rule. It is better practice, however, to purify the ethylene dichloride with Norit. Filter twice to remove the Norit through a double thickness of ordinary filter paper previously washed with purified ethylene dichloride. All carbon particles must be removed.

(c) *Lactic Acid, 85 Per Cent* (Mallinckrodt U.S.P. XII).—Purify by shaking with ethylene chloride in a separatory funnel. Then shake the lactic acid with a small amount of Norit, which is removed by filtering twice through a Buchner funnel. The manifest fluorescence of the lactic acid should not be more than one-third greater than that of distilled water.

(d) *Disodium Phosphate, Na_2HPO_4 .*—Prepare a 0.2 M solution.

(e) *Fluorometer.*—The Coleman Electronic Photo-fluorometer No. 12 is preferred. See page 235 and Figures 26 and 27.

(f) *Filters.*—Coleman B₇-PC9 filters are used.

The reagents used are tested as follows: Add 10 cc. of purified lactic acid to 20 cc. of purified ethylene dichloride in a glass-stoppered 60-cc. bottle previously rinsed with ethylene dichloride. Shake vigorously for five minutes, decant into a 35-cc. test tube also rinsed with ethylene

* BRODIE, BERNARD B. and UDENFRIEND, SIDNEY: *Jour Biol Chem.* (in press)

dichloride and then centrifugalize for two minutes. Transfer at least 8 cc. of the lactic acid phase to a fluorometer tube and compare the fluorescence with that of lactic acid which has not been put through this procedure. The galvanometer readings should not differ by more than one-fourth division.

2. *Procedure.*—(a) *Preparation of Unknown.*—Draw 25 cc. of blood by venepuncture using a syringe containing 6 drops of saturated potassium oxalate solution. Transfer immediately to a tube and centrifugalize at 1500 r.p.m. for one hour. This procedure separates the plasma and minimizes the mechanical fracture of leucocytes. Remove the plasma with a syringe and needle using extreme care not to disturb the layer of leucocytes.

Place 10 cc. of plasma in a 60-cc. glass-stoppered pyrex bottle, add 3 cc. of 0.2 M Na_2HPO_4 and 30 cc. of ethylene dichloride, adding the latter down the side of the bottle so as to prevent immediate mixing with resultant formation of a jell.

Shake vigorously for five minutes preferably in a shaking apparatus. Decant into a 50-cc. round-bottomed centrifuge tube and centrifugalize for ten minutes at 2500 r.p.m. to break the emulsion. If a solid jell forms, break it by vigorous stirring with a glass rod and centrifugalize again at a higher speed.

Insert a 20-cc. pipet below the coagulum carefully and withdraw exactly 20 cc. of the ethylene dichloride solution, placing it in a 60-cc. glass-stoppered pyrex bottle previously rinsed with ethylene dichloride. Add 1 cc. of water and 10 cc. of lactic acid, then shake for five minutes. Transfer to a narrow 35-cc. test tube also rinsed with ethylene dichloride and centrifugalize at 2000 r.p.m. for one minute. Transfer at least 8 cc. of the aqueous phase to a fluorometer tube or cuvet and determine its fluorescence in relation to that of the standard.

(b) *Preparation of Standard and Blank.*—Add 1 cc. of the working atabrine standard, containing 1 gamma of atabrine, to 10 cc. of lactic acid in a fluorometer tube. Mix thoroughly with a chemically clean glass rod.

For the blank use 1 cc. of water and 10 cc. of lactic acid.

(c) *Measurement of Fluorescence.*—Turn on the instrument with filters in place and make the preliminary adjustments as given on page 238.

While the instrument is coming to balance the specimens in the fluorometer tubes are brought to constant room temperature by immersing them in a water bath kept at room temperature.

Read the unknowns with repeated checking of blank and standard by the procedure on page 238. Should any specimen give a reading over 100, the standard is set to a lower value with the sensitivity control and the unknown again read.

The fluorophotometer may show a gradual loss of sensitivity. It can usually be restored by blowing dry air through it for half an hour. This removes moisture from lenses and condenser. It is good practice to do this routinely several times a week.

If this does not restore the sensitivity, check the batteries with a voltmeter. Change any that are low.

If batteries are satisfactory, change the ultra-violet lamp as described in the circular accompanying the instrument.

TABLE 24—NORMAL VALUES OF BLOOD

(All amounts are in milligrams per 100 cc. of whole blood unless otherwise stated. These values are for bloods taken in the morning after a fast of at least ten hours. It should be borne in mind that normal values are dependent to a certain extent upon the methods used, and the values given below are valid only for the methods described herein.)

	<i>Normal</i>	<i>Remarks</i>
Non-protein nitrogen	25 to 35	During digestion there is a rise of about 4 mg. per 100 cc. Anything below 30 mg. is to be considered normal, but values up to 35 mg. are to be found without any evidence of kidney retention.
Urea nitrogen	10 to 15	During digestion of a full meal containing meat a rise of 2 or 3 mg. occurs.
Creatinine	1 to 2	In a selected series of normals the upper limit may be as low as 1.7 mg.; 2 mg. is the more common upper limit of normal.
Uric acid	2 to 4	The figures are based upon the indirect method and are about 1 mg. higher than obtained by the direct method where the uric acid is separated from interfering material.
Sugar	80 to 120	During the absorptive period after food there is a marked increase, dependent on the carbohydrate of the food. The extent of this rise after a standard carbohydrate meal is the basis of the "sugar tolerance test."
Cholesterol	140 to 190	The normal values for cholesterol are dependent upon the method used to a greater extent than other substances. The figures given herein are based upon direct chloroform extraction, but when alcohol-ether extraction is used the upper limit of normal is about 250.
Calcium	10 to 12	These values are for the serum alone and represent the total calcium present in the serum after clotting and separation of the clot.
Phosphorus	3 to 4.5	These values are for the inorganic phosphorus of the serum after separation from the clot. Values for children are higher.
Phosphatase, alkaline	2 to 9	These values are in Bodansky units, although the upper limit of normal is higher than the value given by Bodansky because of the higher pH at which the phosphatase activity is determined.
Phosphatase, acid	0 to 1.1	Values are in Shinowara-Jones-Reinhart units. An increase is reported in carcinoma of the prostate with bone metastases.
Chlorides	450 to 500	Figures for the plasma are somewhat higher than those for whole blood: 575 to 625 mg. per 100 cc.
Alkali reserve (CO ₂ combining power)	55 to 77 vol. %	
Bilirubin	0.25 to 0.8	These values are those obtained with the Evelyn photoelectric method. Earlier methods based upon the less accurate visual colorimetry gave normal values of 0.1 to 0.25.
Icterus index	4 to 6	An icterus index below the normal limit of 4 has so far been found only in cases of secondary anemia. An icterus index of 15 is necessary for jaundice to be evident clinically. Hence, an index between 6 and 15 is termed "latent jaundice."
Total serum proteins	6.5 to 8.0 %	Low in nephritis with edema.
Serum albumin	4.0 to 6.0 %	Low in nephrosis.
Serum globulin	1.5 to 2.5 %	In lipoid nephrosis the globulin is usually normal and the reduction is in serum albumin giving an inverse ratio.
Fibrinogen (plasma)	0.3 to 0.6 %	
Albumin-globulin ratio	2:1 to 2.3:1	

3. Calculations.—The calculation of the quinine concentration of the unknown filtrates is by simple proportion. In a 1 to 50 plasma dilution using a standard containing 1 mg. per liter

$$\frac{R_u}{R_s} \times \frac{1}{21} \times 50 = \text{mg quinine per liter of plasma.}$$

CHEMICAL METHODS FOR VITAMINS

I. Determination of Vitamin A in Serum or Plasma (Kimble).—Vitamin A is extracted from the serum by means of petroleum ether and alcohol. Antimony trichloride in chloroform solution reacts with the vitamin to give a blue color. This color is highly evanescent, reaching a maximum in from two to fifteen seconds and then rapidly fading. For this reason only a photoelectric colorimeter with a well-damped, quick-acting galvanometer can be used. The same reaction is given by carotene which is measured independently and the proper correction value subtracted.

1. Reagents.—(a) *Petroleum Ether*, redistilled, boiling point 30° to 45° C.

(b) *Chloroform*.—Use a good grade of anesthetic chloroform. Do not redistil since chloroform contains
 f
 tive. Redistillation will result in
 decomposition products which will destroy vitamin A. If chloroform con-
 tains water this may be removed by shaking with anhydrous potassium

of A.C.S. or reagent
 flask. Dilute to the

2. Procedure.—

centrifuge tube.
 leum ether. Stopper and shake vigorously for five minutes.
 for three minutes at a speed of 1700 to 2200 r.p.m.

Place 10 cc. of this petroleum ether extract in a colorimeter tube and read the yellow color of the carotene against a petroleum ether blank using
 and the galvanometer reading

With a filter giving a wave length of 620 mμ
 read 100 using a blank of 1 cc. of chloroform plus 9 cc. of the antimony
 trichloride reagent. To the tube containing the vitamin A add 1 cc. of
 chloroform. Place this tube in the instrument, and rapidly add 9 cc. of
 antimony trichloride reagent down the side of the tube from a quick-
 delivery pipet. For this purpose a 10-cc. transfer pipet may be cut off
 at the end and recalibrated. The galvanometer will oscillate briefly as
 mixing occurs, and then move to the left to indicate a point of equilibrium
 which is transitory, but unmistakable. Then it will reverse itself and
 slowly move off again to the right as the color begins to fade. The gal-
 vanometer reading at the maximum color is recorded as G_{max} and its cor-
 responding optical density as D_{max} . The maximum may persist for several
 seconds or for only one or two seconds.

3. Calculation.—When the Evelyn photoelectric colorimeter is used the formula is as follows:

$737 \times [D_{420} - (0.14 D_{440})] = \text{International Units per 100 cc. of serum or plasma.}$ 0.14 D_{440} represents the correction for the carotene. The above factor is for a 1:100 dilution. Corbet

When a colorimeter other than the Evelyn is used, the calibration factor may be obtained by using a sample of reference U.S.P. cod liver oil which usually has a potency of 3000 I.U. per gm. This is suitably diluted with petroleum ether. The carotene is measured with filter 440, and after evaporation of the petroleum ether, the blue antimony trichloride color is measured in the same manner as for the blood serum. From the known value for the cod liver oil the necessary factor is obtained to convert the density reading of the photoelectric colorimeter to International Units.

4. Vitamin A Content of Normal Blood.—For males the average normal value is 131 I.U. with a range of 87 to 190. For females the average normal value is 90 I.U. with a range of 64 to 165.

NOTE.—The vitamin A is present in the blood in a relatively stable form and prompt analysis is therefore unnecessary. If necessary the blood may be kept for several days in a refrigerator before analysis.

II. Determination of Thiamine Hydrochloride (Hennessy and Cerecedo with modifications from Friedemann and Kmiecik).—In the original method of Hennessy and Cerecedo¹ the blood was not deproteinized, but after dilution with acetic acid it was passed through a column of zeolite ion exchange adsorbent. This column frequently clogged up, delaying the filtration and at the same time the adsorbed protein tended to make the results high.

By modifying the method by introducing the metaphosphoric acid precipitation of proteins from Friedemann and Kmiecik² satisfactory results are obtained.

I. Reagents and Apparatus.—(a) *Hydrochloric Acid*.—Approximately 1 N.

(b) *Sodium Hydroxide*.—An approximately 1 N solution and a 15 per cent solution.

(c) *Metaphosphoric Acid*.—A 10 per cent solution is prepared at frequent intervals, about once a week. Since the acid deteriorates rapidly at room temperature, it should be dissolved in cold water and the solution kept in the refrigerator when not in use.

(d) *Takadiastase or Clarase Powder*.

(e) *Bromphenol Blue*.—A 0.1 per cent solution in 50 per cent alcohol.

(f) *Ion Exchange Adsorbent*.—Activated artificial zeolites, such as Decalso or Permutit, 60- to 80-mesh. The zeolite is activated by covering the material in a beaker with 5 per cent acetic acid and boiling gently with constant stirring. The zeolite is allowed to settle and the supernatant liquid decanted. Repeat this procedure three additional times. Then wash with four portions of 25 per cent potassium chloride solution, and finally with boiling distilled water until all the potassium chloride has been removed as indicated by testing the decanted wash water with silver

¹ Jour. Am. Chem. Soc., 61, 179, 1939; Indust. and Engin. Chem., Anal. Ed., 13, 216, 1941.

² Jour. Lab. and Clin. Med., 28, 1262, 1943.

nitrate. The prepared zeolite can then be stored either dry or under water until needed.

(g) *Acid Potassium Chloride Solution*.—Prepare a 25 per cent solution of potassium chloride in 0.1 N hydrochloric acid

(h) *Potassium Ferricyanide Solution*.—Prepare a 1 per cent aqueous solution from reagent grade potassium ferricyanide. This solution is stable under ordinary conditions and may be kept until completely used if stored in an amber colored bottle in a cool dark place.

(i) *Oxidizing Reagent*.—A mixture consisting of 8 parts of 15 per cent NaOH and 2 parts of 1 per cent solution of potassium ferricyanide is prepared just before use.

(j) *Isobutyl Alcohol*.—If the fluorescence of the alcohol is greater than that of distilled water, it must be acidified with concentrated HCl, dehydrated with anhydrous sodium sulfate, and redistilled before use.

Stock solution. Exactly 50 mg. of anhydrous in a desiccator, are dissolved

and made up to exactly 500 cc. in a volumetric flask with a 25 per cent solution of ethyl alcohol in approximately 0.01 N HCl. This solution contains 100 micrograms of thiamine hydrochloride per cc. and if kept in the

solution into a of 0.1 N HCl.

tains 10 micro-

grams of thiamine hydrochloride per cc. and is stable for a few weeks if it is kept in a refrigerator.

(3) *Working solution*, prepared fresh each day. Dilute 1 cc. of the intermediate solution to 100 cc. with 0.01 N HCl. This solution contains 0.1 microgram of thiamine hydrochloride per cc.

(m) *Stopcock Lubricant*.—Heat 3 parts of starch and 2 parts of glycerol to 140° C. with constant stirring and then cool. This lubricant is free of any fluorescent material.

(n) *Fluorophotometer*.—Any sensitive fluorophotometer, such as Coleman, Pfaltz and Bauer, Klett, or Lumetron. The description below applies to the Coleman fluorophotometer.

(o) *Base Exchange Tubes*.—See Figure 28. Each absorption tube is attached by means of a one-hole rubber stopper to a 125-cc. glass suction flask. The tubes are prepared for use by introducing into the bottom of the stem a small roll of glass wool and filling the stem with the activated zeolite. In order to assure rapid drainage distilled water is passed through the tubes just before use.

(p) *Reaction Vessels*.—Special 35-cc. separatory funnels which fit into the standard 50-cc. trunnion cups of the usual centrifuge. See Figure 28.

2. *Procedure*.—The procedure is based upon the fact that the amount of thiamine hydrochloride, within the limits occurring in blood, is directly proportional to the intensity of the fluorescence given by its oxidation product, thiochrome. This linear function is valid for only a limited range of concentrations. However, in the case of blood where the range of concentrations is relatively narrow, the error introduced by assuming linearity will be negligible.

A large part of the thiamine in the blood exists in the form of its pyro-

phosphoric acid ester. As the oxidized product of this form does not give the same amount of fluorescence as that of an equivalent quantity of the thiamine hydrochloride, the pyrophosphoric acid ester must be converted to the free thiamine by an enzymatic hydrolysis at a pH of 4.5 to 5.5 by means of the enzymes takadiastase, clarase, amylase, etc. The soluble proteins are then precipitated by metaphosphoric acid and the thiamine separated from the other substances in the blood by its substitution at a pH of 3 to 3.5 for the potassium ion in activated zeolite. The thiamine is removed from the zeolite with acid KCl and an aliquot oxidized to thiochrome with potassium ferricyanide in an alkaline medium. For a standard a given amount of thiamine hydrochloride is added to a second aliquot and then oxidized. A third aliquot to which the potassium ferricyanide is not added is used as a blank to account for the fluorescent material other than thiochrome which may be present in the blood.

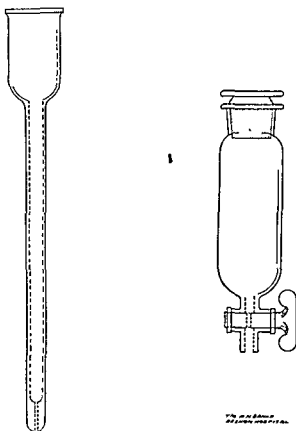


FIG 28—Special glassware for vitamin determinations.

Mix 5 cc. of oxalated blood with 25 cc. of water in a large heavy-walled test tube such as is used in the determination of non-protein nitrogen. Add 0.5 cc. of 1 N HCl, mix by means of a long thin-footed glass rod. Add 0.2 to 0.25 gm. of takadiastase and incubate the mixture one to one and a half hours in a water bath at 40° to 45° C. during which time the contents are frequently mixed by stirring. The mixture is then further acidified with 1.5 cc. of 1 N HCl, after which it is heated ten minutes in a boiling water bath with frequent stirring, and then cooled.

Next add 10 cc. of metaphosphoric acid solution. Adjust the volume to the 50-cc. mark and thoroughly mix the contents. Centrifugalize for ten minutes at high speed.

Take a 45-cc. aliquot of the supernatant liquid, add 2 drops of bromophenol blue indicator and adjust the reaction to 3 to 3.5 by means of 1 N NaOH. Pass the extract through the base exchange tube containing the activated zeolite at the rate of about 1 cc. per minute. After passage of all the extract, the base exchange tube is rinsed with several successive portions of distilled water of about 10 cc. each. The fluid is allowed to drain completely after each of the washings. The thiamine is eluted by passing 20 to 23 cc. of the acid KCl through the column at a rate of 1 cc. per minute. The eluate is collected in a tube graduated at 25 cc. and after collection is made up to the mark with acid KCl and thoroughly mixed.

Three aliquots of 8 cc. each are measured into 3 reaction vessels numbered 1, 2, and 3. To No. 2 add 0.1 microgram of thiamine hydrochloride. To Nos. 1 and 2 add 5 cc. of the oxidizing reagent followed immediately by 10 cc. of isobutyl alcohol. To No. 3 add 5 cc. of 15 per cent NaOH and 10 cc. of isobutyl alcohol. The tubes are shaken for one and one-half minutes and then centrifugalized for three minutes at a speed of about 1100 r.p.m. The aqueous layer is drawn off and discarded and 1 gm. of anhydrous Na_2SO_4 added. The tubes are shaken until the alcohol is perfectly clear. The alcohol is decanted off into the instrument tubes and the fluorescence measured in the fluorophotometer.

Adjust the instrument so that the blank (sample No. 3) gives a zero reading. The blank changes rapidly, all steps from oxidizing to t as rapidly as possible, working with only

3. **Calculation.**—The difference between the readings of Nos. 1 and 2 gives the scale divisions equivalent to 0.1 microgram of thiamine hydrochloride.

$$\frac{0.1}{R_1 - R_2} \times R_1 = \text{micrograms of thiamine hydrochloride in the aliquot taken.}$$

$$\frac{0.1}{R_1 - R_2} \times R_1 \times 69.4 = \text{micrograms of thiamine hydrochloride in 100 cc. of blood.}$$

4. Thiamine Hydrochloride Content of Normal Whole Blood.—

	Women	Men
Range, micrograms per 100 cc.	3.0 to 9.2	3.8 to 11.2
Average, micrograms per 100 cc.	5.6	5.7

NOTES.—As the thiamine content changes gradually even on storage of the sample in the cold, the blood should be stored in the refrigerator and the analysis made as soon as possible, preferably within a few hours of collection. A blank is run on each new lot of reagents following the same procedure as for the blood with the exception that 5 cc. of distilled water are added in place of the blood. If the blank on the reagents is no greater than the reading given by a sample of isobutyl alcohol, it need not be repeated with a given lot of reagents.

The size of the cuvetts and the aperture of the fluorometer determine the quantity of isobutyl alcohol to be used in the extraction, the volume

being kept to a minimum to obtain the maximum sensitivity. When the Coleman instrument is used, 10 cc. of alcohol are sufficient for the extraction as the cuvet requires only 8 cc. of fluid. With other instruments 13 or 15 cc. may be needed.

III. Determination of Ascorbic Acid in Blood Plasma (Mindlin and Butler).

1. Reagents.—(a) *Metaphosphoric Acid Solution*.—A 5 per cent solution is made up freshly at least every two weeks and stored in the icebox.

(b) *2,6-Dichlorophenol-indophenol Solution*.—Dissolve approximately 2.5 mg. in 100 cc. of distilled water. This solution keeps at least three weeks if stored in a dark bottle in the icebox.

(c) *Sodium Acetate Solution*.—Dissolve 4.53 gm. of $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ in distilled water and make up to 100 cc. Adjust to pH 7 with 0.26 cc. of 0.5 M acetic acid. Add a few drops of toluene as a preservative.

(d) *Indophenol-Acetate Solution*.—Mix equal volumes of the sodium acetate and 2,6-dichlorophenol-indophenol solutions. Enough for several days may be made up at a time and kept in the icebox.

2. Procedure.—Collect 5 cc. of oxalated blood in the usual manner. Care must be used to avoid hemolysis. Even small traces of hemolysis will result in rapid destruction of almost all of the ascorbic acid. Centrifugalize and measure 2 cc. of the plasma into a small flask or tube. Add 2 cc. of distilled water and 4 cc. of 5 per cent metaphosphoric acid. Shake gently and filter. The filtrate must be crystal clear.

The galvanometer of the photoelectric colorimeter is set to 100 with a blank consisting of 4 cc. of the indophenol-acetate solution plus 4 cc. of 2.5 per cent metaphosphoric acid solution. A small amount of crystalline ascorbic acid, sufficient to decolorize the dye is added.

To the standard tube add 4 cc. of the indophenol-acetate solution plus 4 cc. of 2.5 per cent metaphosphoric acid. Read in the colorimeter against the blank.

In the unknown tube place 4 cc. of indophenol-acetate solution and 4 cc. of the metaphosphoric acid filtrate. Mix at once and read in colorimeter at thirty seconds.

3. Calculations.—The concentration of ascorbic acid in mg. per 100 cc. of plasma equals $K (\log G_1 - \log G_2)$ where G_1 is the reading of the unknown, G_2 is the reading of the blank, and K is a constant for a given solution containing no ascorbi

ing known amounts of ascorbic acid in the colorimeter after treatment as in the procedure above. For the Evelyn instrument the value for K is 8.6.

NOTE.—The strength of the indophenol-acetate solution plus metaphosphoric acid must be such that the reading of the galvanometer will be between 50 to 55. The proper filter to be used in the colorimeter is one transmitting wave length 520 $m\mu$ and the value for K given above is for use with this filter. The reading of the sample must be less than that of the blank, otherwise all of the dye has been reduced by the sample. The amount of dye is sufficient to take care of a concentration of 2.5 mg. of ascorbic acid per 100 cc. of plasma. Analysis should be started within one hour from the time the sample was taken, otherwise decomposition will occur.

QUANTITATIVE CHEMICAL ANALYSIS OF THE CEREBROSPINAL FLUID

In general, the methods used for the chemical analysis of blood may be applied with necessary modifications to the analysis of cerebrospinal fluid. Only those procedures most frequently done are considered here. All specimens for chemical examination must be free of blood if results are to be of value.

I. Determination of Sugar (Folin-Wu, Modified).—1. Reagents.—The reagents are the same as those required for a blood sugar determination, page 207.

2. Procedure.—To 1 cc. of cerebrospinal fluid add 3 cc. of distilled water, 0.5 cc. of sodium tungstate and 0.5 cc. of 0.66 N sulfuric acid. Allow to stand for ten minutes, and then filter.

The test is completed as for the blood sugar, page 208.

3. Calculation.—When the lower standard is used, the reading of the standard, usually 20 mm., multiplied by 50 and divided by the reading of the unknown equals mg. sugar per 100 cc. of spinal fluid. When the higher standard is used substitute 100 for 50 in the above.

II. Determination of Chlorides.—1. Reagents.—The same as those used in blood chloride determinations.

2. Procedure.—A protein-free filtrate of cerebrospinal fluid is prepared in the same manner as in the determination of sugar. Transfer 2 cc. of this filtrate to a porcelain evaporating dish, add 3 cc. of water and titrate in the same manner as for blood chlorides.

3. Calculation.—The titer times 125 equals mg. of chlorides, as sodium chloride, per 100 cc. of cerebrospinal fluid.

III. Determination of Protein.—A. Colorimetric Method (Johnston and Gibson).—This method is based upon the tyrosine content of the protein. The color developed by the reaction of the tyrosine of the protein with Folin's phenol reagent is compared with that of a standard tyrosine solution treated in the same manner.

1. Reagents.—(a) *Trichloroacetic Acid*, 20 per cent.

(b) *Sodium Hydroxide*, 10 per cent.

(c) *Folin's Phenol Reagent.*—Transfer 100 gm. of sodium tungstate and 25 gm. of sodium molybdate together with 700 cc. of water to a 1500-cc. Florence flask. Add 50 cc. of 85 per cent phosphoric acid and 100 cc. of concentrated hydrochloric acid. Connect with a reflux condenser by means of a cork stopper wrapped in tinfoil. Boil gently for two hours. At the end of this period, cool, transfer to a liter volumetric flask and dilute to the mark with distilled water. Store in glass-stoppered bottles.

(d) *Tyrosine Standard Solution.*—Dissolve 200 mg. of pure dry tyrosine in 1 liter of 0.1 N HCl.

(e) *Sodium Carbonate*, 25 per cent.

(f) *Lithium Sulfate*, 2 per cent solution.

2. Procedure.—To 2 cc. of spinal fluid in a 15-cc. centrifuge tube, add 3 cc. of distilled water and 1 cc. of 20 per cent trichloroacetic acid. Mix by twirling. Allow to stand two to three hours, or overnight for flocculation and then centrifugalize at high speed for three minutes. Pour off the supernatant fluid and drain. Add 0.25 cc. of 10 per cent NaOH and heat in a

boiling water bath for ten minutes. Add 3 cc. of 2 per cent lithium sulfate, 0.5 cc. of the phenol reagent, 1.5 cc. of 25 per cent Na_2CO_3 , and dilute to the 6-cc. mark with distilled water. Mix by inversion, allow to stand ten minutes for color development, and compare with the nearest standard with the standard set at 10 mm.

Three standards should be prepared as follows:

No. 1—0.5 cc. of standard tyrosine, 6 cc. of 2 per cent lithium sulfate, 1 cc. of phenol reagent, 3 cc. of 25 per cent Na_2CO_3 , water to 12 cc.

No. 2—1 cc. of tyrosine standard, 6 cc. of 2 per cent lithium sulfate, 1 cc. of phenol reagent, 3 cc. of 25 per cent Na_2CO_3 , water to 12 cc.

No. 3—2 cc. of tyrosine standard, 6 cc. of 2 per cent lithium sulfate, 1 cc. of phenol reagent, 3 cc. of 25 per cent Na_2CO_3 .

3. Calculation.—With the standard set to read 10 mm. the factors for the different standards are as follows:

No. 1—285 divided by the reading of the unknown equals mg. protein per 100 cc. spinal fluid.

No. 2—570 divided by the reading of the unknown equals mg. protein per 100 cc. spinal fluid.

No. 3—1140 divided by reading of unknown equals mg. protein per 100 cc. spinal fluid.

B. Turbidimetric Method (Denis and Ayer, Modified).—If a photoelectric colorimeter is available the measurement of the turbidity produced when sulfosalicylic acid is added to spinal fluid is the simplest and most rapid method for the quantitative measurement of the protein content. The method is sufficiently accurate for clinical use.

1. Reagents.—(a) *Sulfosalicylic Acid*, 3 per cent solution.

(b) *Sodium Chloride*, 0.85 per cent (physiological saline).

2. Procedure—Into a test tube pipet exactly 1 cc. of cerebrospinal fluid. The fluid should be clear and free from any trace of blood. Add 4 cc. of 3 per cent sulfosalicylic acid. Mix contents thoroughly by shaking. Allow to stand ten minutes, again mix by shaking and read in a photoelectric colorimeter using a filter that will give a wave length of approximately 420 m μ . The 100 per cent setting of the colorimeter is obtained against a blank consisting of 1 cc. of distilled water plus 4 cc. of the sulfosalicylic acid solution. The value of the unknown is obtained by reference to a calibration curve prepared as described below.

3. Preparation of the Calibration Curve.—Obtain a sample of normal human serum. The sample should be taken before breakfast and be clear and free from excess fat. Determine the protein content of this serum by a micro- or macro-Kjeldahl method. Prepare a series of about 10 dilutions of this serum, using 0.85 per cent NaCl for the diluting fluid. The dilutions are so prepared that the protein content varies from 10 mg. per 100 cc. to about 100 mg. per 100 cc. To the diluted serums, using 1 cc. of each, add 4 cc. of the sulfosalicylic acid solution and read in the photoelectric colorimeter in the same manner as for the cerebrospinal fluid. Plot the readings on graph paper against the known protein values of the serum dilutions.

NOTES ON METHOD.—The total amount of fluid available for measurement is 5 cc. Should the cuvetts of the colorimeter require more than this

the quantities may be doubled, that is 2 cc. of cerebrospinal fluid plus 8 cc. of the sulfosalicylic acid solution.

If the concentration of the fluid exceeds 100 mg. protein per 100 cc. the sample should be diluted and the value obtained multiplied by the dilution factor.

Any blue filter is satisfactory for the photoelectric measurements. The shorter wave lengths such as 420 $m\mu$ will give greater sensitivity than longer wave lengths.

IV. Determination of Sulfonamides.—Sulfonamides are determined in exactly the same manner as described for the blood, page 219. No correction, however, for loss occurring as a result of the precipitation of the proteins is required with spinal fluid.

TABLE 25 —NORMAL VALUES OF CEREBROSPINAL FLUID
(All in mg. per 100 cc)

	<i>Normal</i>	<i>Remarks</i>
Sugar	40 to 70	Decreased in tuberculous meningitis and acute suppurative meningitis. Increased in epidemic encephalitis and certain forms of syphilis of the central nervous system.
Total protein	20 to 40	Normal values are affected to a large extent by method of analysis. Methods based upon turbidity measurement may give normals up to 100.
Chlorides	700 to 750 as NaCl	Serum protein dilution without chloride change reduces spinal fluid chlorides. Dehydration raises the chloride content of spinal fluid. Elevation of spinal fluid protein as in spinal tumors diminishes the chloride.

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becomes fatigued. Tests should be made in a room free from interfering odors.

1. *Procedure.*—(a) *Cold Odor.*—Shake the collecting bottle vigorously while it is one-half to two-thirds full of the sample of water at room temperature. Remove the stopper and note the odor at the mouth of the bottle. Repeated shaking and smelling will reduce the odor of the sample and should be avoided.

(b) *Hot Odor.*—Place about 150 cc. of the sample in a 500-cc. Erlenmeyer flask. Cover the mouth of the flask with a watch-glass and heat to approximately 65° C. Agitate with a rotary motion, slip the cover to one side, and quickly sniff the odor.

II. *Color.*—The color of water is usually of vegetable origin, from dead leaves, roots, bark, etc. "Color" means the "true color," or that due solely to the substances in solution. The "modified by the matter in suspension" in water containing matter in suspension to remove suspended matter gives the best results. Filters cannot be used,

($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), and dilute to 1 liter with distilled water. This solution contains 0.5 gm. of platinum and 0.25 gm. of cobalt, and has a color of 500.

Prepare standards by placing in 50-cc. Nessler tubes the following quantities of the standard color solution:

Color 5	0 5 cc.
Color 10	1 0 cc.
Color 15	1 5 cc.
Color 20	2 0 cc.

and so on up to color 70, filling each tube to the mark with distilled water. These standards keep well if protected against dust and evaporation.

2. *Procedure.*—Compare 50 cc. of the water under examination in a Nessler tube with these standards, viewing them vertically with reflected light over a white surface. Waters having a color greater than 70 must be diluted with distilled water and the color obtained multiplied by the dilution. The Nessler tubes used must be of clear glass and equal in height at the 50-cc. mark.

For field work, the glass disc apparatus of the U. S. Geological Survey may be used.

3. *Results.*—Report colors between 1 and 50 to the nearest unit; between 51 and 100 to the nearest five; between 101 and 250 to the nearest ten; and between 251 and 500 to the nearest twenty.

III. *Turbidity.*—The turbidity of water is due to suspended matter such as clay, silt, similar materi. per million of water.

A. *Fuller's Earth Method.*—1. *Reagents.*—(a) *Stock Suspension.*—Add about 5 gm. of fuller's earth to 1 liter of distilled water, agitate intermittently for an hour and then allow to stand twenty-four hours. With-

draw the supernatant without disturbing the sediment. Determine the turbidity of this suspension, preferably with a Jackson candle turbidimeter. If this instrument is not available, the platinum wire method given under "B" may be used, but it is much less accurate.

(b) *Standards for Comparison.*—Prepare standards for comparison from this stock suspension by dilution with distilled water. Standards of 0, 5, 10, 15, and 20 parts per million are prepared in clear glass bottles of 1 liter capacity or greater, leaving enough free space in the bottle to allow adequate agitation. Standards of 20, 30, 40, 50, 60, 70, 80, 90, and 100 may be prepared in smaller bottles or 100-cc. Nessler tubes. These standards may be preserved by adding a small amount of mercuric chloride.

2. *Procedure.*—Fill a clear glass bottle of the same size and shape as those holding the standards with the specimen or place 100 cc. in a 100-cc. Nessler tube, as the case requires. Shake the specimen and standards thoroughly, and view sidewise toward the light, looking directly at some object and noting the distinctness of its outline. The standard with which the sample compares represents the degree of turbidity.

3. *Expression of Results.*—Turbidity results are expressed as follows:

4. *Coefficient of Fineness.*—The quotient obtained by dividing the weight of the suspended matter in the sample by the turbidity, both expressed in the same unit (p.p.m.) gives the coefficient of fineness. When this quotient is greater than unity, the suspended matter is coarser, where less than unity, finer than are the particles of the standard.

B. *Platinum Wire Method.*—Item No. 44650, Medical Department Supply Catalog, U. S. Army, is a device for the determination of both color and turbidity. The turbidity scale consists of a platinum wire 1 mm. in diameter inserted in a metallic rod. This is suspended in the water until the platinum wire just disappears from view, and the depth of immersion read directly on the scale as turbidity in p.p.m. Although this method is subject to quite a large error, it is well adapted for field determinations.

CHEMICAL EXAMINATION OF WATER

The sanitary chemical analysis of a water may serve many purposes, among which are the determination of its safety, palatability, and general suitability for domestic use, the tracing of the past history of the water, the supervision of measures directed toward its purification, and the determination of its suitability for various industrial uses. Many of the substances for which tests are made are not themselves objectionable, but are indicative of unsatisfactory or dangerous conditions.

The following determinations are usually made:

Ammonia nitrogen	Alkalinity
Nitrite nitrogen	pH value
Nitrate nitrogen	Hardness
Chlorides	Minerals as requested
Chlorine	Dissolved oxygen
Carbon dioxide	Solids, total, volatile and fixed

In addition search must occasionally be made for toxic substances such as the heavy metals, phenol, fluorides, and possibly chemical warfare agents.

The nitrogen determinations measure the successive steps of organic decomposition and inorganic oxidation. The conversion of nitrogen from the organic to the inorganic or mineral state is known as nitrification, and is brought about by the so-called nitrifying bacteria. The first step in the decomposition of organic matter produces nitrogen in the form of ammonia. The next step is oxidation to nitrite and the final step, oxidation of the nitrite to nitrate.

1. Ammonia Nitrogen.—Free ammonia is recovered quantitatively when the water is distilled at a pH of 7.4. This pH value is maintained by the addition of a buffer. Direct nesslerization is used for waters high in ammonia and for sewages.

1. Apparatus.—Distillation is carried on in a glass flask with a vertical condenser arranged so that the distillate drops directly into the receiving vessel. Nessler tubes of clear colorless glass with polished bottoms and graduated to contain 50 cc. are used to collect the distillate. The graduation marks on the tubes should be 20 to 25 cm. from the bottom; in a matched set, the marks on all tubes should agree within a range of 6 mm.

2. Reagents.—(a) *Ammonia-free Water.*—Add about 0.3 gm. per liter of anhydrous sodium carbonate to distilled water, and redistil until the distillate is free from ammonia. Cool and place the water remaining in the flask in a glass-stoppered bottle previously rinsed with ammonia-free water. It may also be prepared by redistilling distilled water containing 10 cc. of concentrated sulfuric acid per liter, and collecting the distillate.

(b) *Nessler Reagent.*—Dissolve 50 gm. of potassium iodide in about 35 cc. of cold ammonia-free water. Add, with constant stirring, a saturated solution of mercuric chloride until a slight precipitate persists. Add 400 cc. of 9 N sodium or potassium hydroxide, clarified by sedimentation. Dilute to 1 liter, allow to clarify, and decant. The reagent should give the characteristic color with ammonia within five minutes, and should not produce a precipitate with small amounts of ammonia within two hours.

(c) *Standard Ammonium Chloride Solution.*—Dissolve 3.818 gm. of ammonium chloride in distilled water and dilute to 1 liter.

(d) *Phosphate Buffer Solution, 0.5 M.*—Dissolve 14.3 gm. of monobasic potassium phosphate (KH_2PO_4) and 90.15 gm. of dibasic potassium phosphate ($\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$) in sufficient distilled water to make 1 liter of solution.

3. Preparation of Permanent Standards.—(a) *Reagents.*—(1) Potassium chloroplatinate solution: Dissolve 2 gm. of the salt in a small volume of distilled water, add 100 cc. of concentrated hydrochloric acid and dilute to 1 liter with distilled water.

(2) Cobaltous chloride solution: Dissolve 12 gm. of the dry crystals ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) in a small volume of distilled water, add 100 cc. of concentrated hydrochloric acid and dilute to 1 liter with distilled water.

(b) *Preparation of Standards.*—Measure into 50-cc. Nessler tubes the appropriate volume of the solutions just described as shown in Table 27; dilute to the mark, and mix.

The values given in the table are approximate only; the actual equivalents of standards so prepared will differ with the quality of the Nessler reagent and the color sensitivity of the observer's eye. They should be

compared with nesslerized ammonia standards and the tints modified as necessary. Such comparison should be made for each newly prepared Nessler solution and checked by each individual using the standards.

TABLE 27.—PERMANENT STANDARDS FOR AMMONIA NITROGEN

Value in ammonia nitrogen, mg.	Volume of platinum solution, cc.	Volume of cobalt solution, cc.
0 000	1 2	0 0
0 001	1 8	0 0
0 002	2 8	0 0
0 004	4 7	0.1
0 007	5.9	0 2
0 010	7.7	0 5
0 014	9 9	1 1
0 017	11 4	1 7
0.020	12.7	2 2
0 025	15 0	3 3
0 030	17 3	4 5
0 035	19 0	5 7
0 040	19 7	7 1
0 045	19 9	8 7
0 050	20 0	10 4
0 060	20 0	15 0
0 070	20 0	22 0

4. Procedure.—Free the apparatus from ammonia by placing in it about 500 cc. of distilled water, and boiling until the distillate shows no trace of ammonia. Empty the flask and place in it 500 cc. of the sample or a smaller measured volume diluted to 500 cc. with ammonia-free water. Add 10 cc. of phosphate buffer solution and distil at a rate of not more than 10 cc. nor less than 6 cc. per minute. Collect the distillate in 50-cc. Nessler tubes, filling each to its mark, until four portions are collected; if the nitrogen is high, the distillate may be collected in a 200-cc. volumetric flask.

Prepare a series of 16 Nessler tubes containing respectively 0.0, 0.1, 0.3, 0.5, 0.7, 1.0, 1.4, 1.7, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0 cc. of standard ammonium chloride solution, and dilute to the 50-cc. mark with ammonia-free water. These standards contain 0.01 mg. of nitrogen for each cc. of the standard solution.

Nesslerize the standards and the four portions of distillate by adding 1 cc. of Nessler solution to each tube. Allow the tubes to stand for ten minutes, and compare. The temperature of standards and prepared sample should be practically the same. Comparison may be made with the permanent standards instead of those prepared from ammonium chloride. If the color in any of the portions is greater than that of the highest standard, mix thoroughly, take an accurately measured fraction, and dilute to 50 cc. in a Nessler tube with ammonia-free water. Make the color comparison, and multiply the reading by the dilution made.

5. Calculation of Results.—The sum of the readings for the four portions is equal to the number of mg. of ammonia nitrogen in the original volume of the sample distilled.

$$\text{P p m NH}_3 \text{ nitrogen} = \text{Mg. NH}_3 \text{ N in sample} \times \frac{1000}{\text{Volume of sample in cc.}}$$

II. Albuminoid Nitrogen.—Albuminoid nitrogen is the nitrogen equivalent formed or liberated from the nitrogen substances in water by alkaline permanganate. The determination has been abandoned as obsolete by many laboratories. It is of value mainly where past records exist by which the results may be interpreted. For directions see Standard Methods of Water Analysis.

III. Organic Nitrogen.—The Kjeldahl method of determining organic nitrogen effects the complete destruction of organic matter with conversion of all nitrogen to ammonia.

1. **Reagents.**—(a) *Sulfuric Acid, Concentrated*, nitrogen-free.

(b) *Sodium or Potassium Sulfate*, anhydrous.

(c) *Sodium Hydroxide*, 10 per cent solution, ammonia-free.

2. **Procedure.**—Boil 500 cc. of the sample in a Kjeldahl flask to remove ammonia; this usually causes a loss of 200 cc. of the sample which may be collected for the determination of ammonia nitrogen. Add 5 cc. of the sulfuric acid and a small piece of ignited pumice. Mix by shaking. Digest under a hood until copious fumes of sulfuric acid are given off and the liquid becomes colorless or a pale straw color. The digestion temperature may be raised by the addition of 5 gm. of anhydrous potassium or sodium sulfate. When digestion is complete, cool and dilute to about 300 cc. with ammonia-free water. Make alkaline with the sodium hydroxide solution, distil off the ammonia and collect and estimate it as directed under ammonia nitrogen, page 256.

IV. Nitrite Nitrogen.—1. **Reagents.**—(a) *Sulfanilic Acid*.—Dissolve 8 gm. of pure sulfanilic acid in a liter of approximately 5 N acetic acid (sp. gr. 1.041).

(b) *Alpha-naphthylamine Acetate*.—Dissolve 5 gm. of solid alpha-naphthylamine in a liter of 5 N acetic acid, and filter through washed absorbent cotton. This solution may be filtered periodically to remove decomposition products.

(c) *Sodium Nitrite Stock Solution*.—Dry pure crystalline sodium nitrite in a desiccator overnight. Weigh out 0.493 gm. and dissolve in a liter of distilled water.

(d) *Standard Sodium Nitrite Solution*.—Dilute 100 cc. of stock solution to 1 liter, then dilute 50 cc. of this dilution to 1 liter with sterilized nitrite-
in a sterilized bottle.
g. NO_2 .

tassium or ammonium
alum in a liter of distilled water. Precipitate the aluminum by adding ammonium hydroxide. Wash the precipitate by decantation until free of chloride, nitrite, and ammonia.

(f) *Fuchsin Solution*.—Contains 0.1 gm. of basic fuchsin per liter.

2. **Procedure.**—Place 50 cc. (or a smaller amount diluted to 50 cc.) of the sample, decolorized if necessary with aluminum hydroxide, in a Nessler tube. Also prepare a series of Nessler tubes containing 0.0, 0.1, 0.2, 0.4, 0.7, 1.0, 1.4, 1.7, 2.0 and 2.5 cc. of standard sodium nitrite solution diluted to 50 cc. Add to all tubes 1 cc. of sulfanilic acid and 1 cc. of alpha-naphthylamine acetate. Mix thoroughly and allow to stand ten minutes but not over thirty minutes; compare the sample with the standards.

Permanent standards of sufficient accuracy for waters high in nitrite and for sewage may be prepared by matching the nitrite standards with

dilutions of the fuchsin solution. The standards should be kept in the dark and checked once a month.

3. Calculation.—

$$\text{P p m. nitrite N} = 0.5 \times \frac{\text{Cc. solution in standard}}{\text{Volume of sample taken in cc.}}$$

V. Nitrate Nitrogen.—Nitrate in waters is ordinarily reported in terms of the nitrogen equivalent, but in mineral analysis it is reported as NO_3 .

A. Phenoldisulfonic Acid Method.—1. **Reagents.**—(a) *Phenoldisulfonic Acid.*—Dissolve 25 gm. of pure white phenol in 150 cc. of pure concentrated sulfuric acid. Add 75 cc. of fuming sulfuric acid (15 per cent free SO_3), stir well, and heat for two hours at about 100°C .

(b) *Potassium Hydroxide Solution.*—Prepare an approximately 12 N solution, 10 cc. of which will neutralize about 4 cc. of the phenoldisulfonic acid.

(c) *Standard Nitrate Solution.*—Dissolve 0.7216 gm. of pure recrystallized potassium nitrate in 1 liter of distilled water. Evaporate 50 cc. of this solution to dryness on the water bath. Moisten the residue quickly and thoroughly with 2 cc. of phenoldisulfonic acid, and rub with a glass rod to insure intimate contact. Dilute to 500 cc.; this is the standard solution, 1 cc. of which contains 0.01 mg. of nitrate nitrogen, or 0.04427 mg. of NO_3 .

(d) *Standard Silver Sulfate Solution.*—Dissolve 4.397 gm. of silver sulfate, free from nitrate, in 1 liter of water; 1 cc. of this solution is equivalent to 1 mg. of chloride radicle.

2. Procedure.—The alkalinity, chloride, nitrite, and color of the sample must first be determined. If the color is high, decolorize with aluminum hydroxide suspension. Measure into an evaporating dish 100 cc. of the sample, or such a volume as will give

Add sufficient 0.02 N sulfuric acid
if the chloride content is above 30 p

to precipitate all but about 0.1 mg. of chloride. Add a little aluminum hydroxide, stir thoroughly, allow to stand for a few minutes, filter, and wash with distilled water. Evaporate the filtrate and washings to dryness, and add 2 cc. of phenoldisulfonic acid, rubbing with a glass rod to insure intimate contact. If the residue becomes packed or appears vitreous, heat the dish on the water bath for a few minutes. Dilute the mixture with distilled water and add the potassium hydroxide slowly until the maximum yellow color is developed. Transfer the solution to a Nessler tube, filtering if necessary and dilute to the mark. Compare with standards made by adding 2 cc. of the potassium hydroxide to measured volumes of standard nitrate solution and diluting to 50 cc. in Nessler tubes. The following values of standard nitrate solution are suggested: 0.1, 0.3, 0.5, 0.7, 1.0, 3, 5, 10, 20, 30, 40 and 50 cc. These standards may be kept for several weeks without deterioration.

If the color of the unknown is more brown than that of the standards it may be clarified by activated carbon as directed on page 250.

3. Calculation of Results.—

$$\text{P p m. nitrate N} = 10 \times \frac{\text{Cc. of standard nitrate solution}}{\text{Volume of sample taken, in cc.}}$$

B. Reduction Method.—1. **Reagents.**—(a) *Sodium or Potassium Hydroxide.*—Dissolve 250 gm. in 1250 cc. of distilled water. Add several strips

of aluminum foil and allow the evolution of hydrogen to continue overnight. Concentrate to 1 liter by boiling.

(b) *Aluminum Foil*.—Use strips of pure aluminum about 10 cm. long, 6 mm. wide, and 0.33 mm. thick.

2. *Procedure*.—To 100 cc. or less of the sample in a 300-cc. casserole add 2 cc. of hydroxide solution and concentrate by boiling to about 20 cc. Place the liquid in a test tube (reduction tube) about 16 cm. long and 3 cm. in diameter. Rinse the casserole and add the rinsings to the tube until the volume is about 75 cc. Add a strip of aluminum foil, and close the tube with a rubber stopper equipped with a trap consisting of a U-shaped glass tube of 5 mm. diameter, having one arm flush with the lower side of the stopper, and the other arm extending below the surface of distilled water in another test tube.

Allow the action to proceed for at least four hours, preferably overnight. Dilute the contents of the tube with 250 cc. of ammonia-free water in a distilling flask, distil, and collect the distillate in a 200-cc. flask. Nesslerize an aliquot part, and compare with ammonia standards.

If the supernatant liquid in the reduction tube is clear and colorless, it may be diluted to a definite volume and an aliquot part nesslerized without distillation.

VI. *Oxygen Consumed*.—This determination is seldom made on waters. For directions, see the section on Sewage, Effluents, etc.

VII. *Residue on Evaporation*.—Total residue on evaporation, or total solids, includes suspended and dissolved solids. It is a rough index of the relative quantities of inorganic and organic solids. The loss in weight on ignition of such a residue represents the amount of organic and volatile material; the weight after ignition represents the inorganic matter.

1. *Total Residue*.—Ignite and weigh a clean platinum or silica dish and add 100 cc. of the thoroughly shaken sample. Evaporate to dryness on a water or steam bath and dry the residue for one hour at 103° C. Cool in a desiccator and weigh. The increase in weight equals the total solids.

$$\text{P p.m. total solids} = \text{Mg. increase in weight} \times \frac{1000}{\text{Cc. of sample taken}}$$

2. *Dissolved Solids*.—Proceed as above using a filtered sample.

3. *Fixed Residue and Loss on Ignition*.—Ignite the residue in the dish at a low red heat, noting color change and production of odor. If great accuracy is desired the ignition should be conducted in an electric muffle furnace at 600° C. Allow the dish to cool, moisten the residue with distilled water, dry at 103° C. and weigh.

VIII. *Suspended Matter*.—1. *Reagent*.—(a) *Asbestos Fiber Cream*.—Prepare a dilute cream of asbestos fiber which has been finely shredded, thoroughly ignited, treated with strong hydrochloric acid for at least twelve hours and washed with distilled water until free from acid.

2. *Procedure*.—Prepare a mat of the asbestos fiber 2 mm. thick in a Gooch crucible. Dry in an oven at 103° C., cool and weigh. Filter 1 liter of samples having a turbidity less than 50 p.p.m.; for samples having a higher turbidity filter enough to obtain 50 to 100 mg. of suspended matter. Dry for one hour at 103° C., cool in a desiccator and weigh.

$$\text{P p.m. suspended solids} = \text{Weight in mg.} \times \frac{1000}{\text{Cc. of sample taken}}$$

Suspended matter may be also calculated from the difference between total solids in filtered and unfiltered samples. The loss on ignition due to suspended matter may be computed from the difference in ignition loss on filtered and unfiltered samples, or determined directly, using an ignited Gooch crucible.

IX. Hardness.—Water which requires an excessive amount of soap to form a lather, or forms much incrustation on vessels in which it stands or is heated is called "hard water." Calcium and magnesium, and iron and aluminum to a less extent, are responsible for these effects.

Hardness, like alkalinity, is expressed in terms of calcium carbonate. The calcium carbonate equivalent of the calcium and magnesium content, sometimes with that of iron and aluminum, is a measure of the total hardness of a water. The soap and soda reagent methods are approximate methods; ordinary quantitative methods have greater potential accuracy. When the total hardness is greater than the carbonate and bicarbonate alkalinity, the amount of hardness equivalent to the alkalinity is called the carbonate hardness and the amount of hardness in excess of this is called the non-carbonate hardness. When the total hardness is equal to or less than the sum of the bicarbonate and carbonate alkalinity the total hardness is all carbonate hardness.

A. Total Hardness by Calculation.—The most accurate method of determining total hardness is by computing it from the results of calcium and magnesium determinations. Iron and other metals must be included in the calculation if present in any quantity. Total hardness as CaCO_3 equals 2.495 Ca plus 4.115 Mg.

B. Total Hardness by the Soap Method.—1. **Reagents.**—(a) *Standard Calcium Chloride Solution.*—Dissolve 0.5 gm. of pure calcite (calcium carbonate) with a little dilute hydrochloric acid, being careful to avoid loss by spattering. Wash down with carbon dioxide-free distilled water and neutralize with ammonium hydroxide to slight alkalinity using litmus as an indicator. Make up to 500 cc. with carbon dioxide-free distilled water and store in a glass-stoppered bottle. One cc. of this solution is equivalent to 1 mg. of calcium carbonate.

(b) *Standard Soap Solution.*—Make up a stock solution by shaking vigorously about 100 gm. of pure powdered castile soap in 1 liter of 80 per cent ethyl alcohol. Let this solution stand at least overnight and decant. This stock solution is nine to ten times as strong as the dilute standard soap solution. Titrate against the standard calcium chloride solution and dilute with 80 per cent ethyl alcohol until 1 cc. of the resulting solution is equivalent to 1 cc. of the standard calcium chloride solution, making a due allowance for a lather factor which is the amount of standard soap solution required to produce a permanent lather in 50 cc. of distilled water. The lather factor should be calculated from a minimum of 5 determinations. It will vary usually from 0.5 to 1.4 cc. with different soaps. Carbon dioxide-free water should be used for lather factor determinations and for standardization of the solution. One cc. of this solution after subtracting the lather factor is equivalent to 1 mg. of calcium carbonate.

(c) *Sulfuric Acid, 0.02 N.*

(d) *Sodium Carbonate, 0.02 N.*

(e) *Phenolphthalein Indicator.*

(f) *Methyl Orange Indicator.*

2. **Procedure.**—Measure 50 cc. of the sample into a 250-cc. bottle. Add the standard soap solution from a buret, in small amounts at a time, shaking vigorously after each addition, until a strong permanent lather is obtained which will stand for at least five minutes. It is usually satisfactory to add amounts equal to the lather factor as the first additions, and then, as the end point is approached to add 0.1 cc. portions. Any false end point should be noted and recorded as this is the dividing line between the calcium and magnesium salts. Acid waters should first be rendered neutral to methyl orange by addition of 0.02 N sodium carbonate. Samples containing appreciable amounts of free carbon dioxide should be neutralized to faint pink with 0.02 N sodium carbonate before testing. If the hardness test requires more than 7 cc. of soap solution, it is best to take an aliquot part and dilute to 50 cc. with carbon dioxide-free water. After deduction of the lather factor, multiply accordingly to obtain the correct result.

3. **Calculation.**—The final buret reading, after deducting the lather factor, multiplied by 20 gives the total hardness as calcium carbonate in p.p.m. The difference between the false end point and the true total hardness indicates the amount of magnesium salts, the balance being calcium salts, all, when multiplied by 20, expressed as p.p.m. of calcium carbonate.

To avoid mistakes in the false or magnesium end point read the buret after the
of soap so
will disap.
sists for less than five minutes.

C. **Non-carbonate Hardness by the Soda Reagent Method.**—The soda reagent methods for non-carbonate and total hardness may be used in place of the soap method for the harder waters.

1. **Reagents.**—(a) *Soda Reagent.*—Dissolve 2 gm. of sodium hydroxide and 2.65 gm. of anhydrous sodium carbonate in distilled water and make up to 1 liter. This solution is approximately 0.1 N.

2. **Procedure.**—Measure 200 cc. of the sample into a 500-cc. pyrex Erlenmeyer flask, and 200 cc. of distilled water into a similar flask. Treat the contents of each flask in the following manner: Boil fifteen minutes to expel free carbon dioxide, then add 25 cc. of soda reagent and again boil for ten minutes.
to the mark with boil
and titrate 50 cc. of each
of methyl orange.

3. **Calculation.**—The non-carbonate hardness in p.p.m. of calcium carbonate is equal to twenty times the difference between the number of cc. of sulfuric acid required for the soda reagent in distilled water and the number required for the soda reagent in the sample.

Water containing bicarbonate and carbonate in excess of calcium and magnesium requires a larger amount of acid to neutralize the sample after it has been treated than is required to neutralize the amount of soda reagent originally added.

D. **Total Hardness by the Soda Reagent Method.**—Add standard sulfuric acid to 200 cc. of the sample until the alkalinity is neutralized. Then apply the non-carbonate hardness method. This gives fairly satisfactory results for total hardness of hard waters.

X. Alkalinity.—The alkalinity of a natural water represents its content of carbonates, bicarbonates, hydroxides and, occasionally, borates, silicates and phosphates. It is determined by titration with a standard solution of a strong acid to certain end points or hydrogen ion concentration. Indicators are selected which show definite color changes at these points.

Dilute bicarbonate solutions have a pH of about 8 and dilute carbonic acid solutions, a pH of about 4. The amount of standard acid required to bring the water to pH 8 measures approximately the hydroxide alkalinity plus one-half the normal carbonate alkalinity, and the amount required to bring it to pH 4 corresponds to the total alkalinity.

Phenolphthalein, cresolphthalein, or thymolsulphonphthalein are satis-

in place of titration with indicators.

1. **Reagents.**—(a) *Sulfuric Acid*—0.02 N solution. Prepare and standardize a stock solution of 1 N sulfuric acid. Make the 0.02 N acid from this by dilution. The stock solution keeps indefinitely in a glass bottle.

(b) *Phenolphthalein Indicator*.—Dissolve 5 gm. of a good quality phenolphthalein in 1 liter of 50 per cent alcohol. Neutralize with 0.02 N sodium hydroxide. The alcohol should be diluted with boiled distilled water.

(c) *Methyl Orange Indicator*.—Dissolve 0.5 gm. of a good grade of methyl orange in 1 liter of distilled water. Keep the solution in the dark.

(d) *Erythrosine Indicator*.—Dissolve 0.1 gm. of erythrosine (the sodium salt) in 1 liter of freshly boiled distilled water.

2. **Procedure.**—(a) *With Phenolphthalein*.—Add 4 drops of the indicator to 50 or 100 cc. of the sample in a white porcelain casserole or an evaporating dish, or in a glass flask over a white surface. If the solution becomes colored, hydroxide or normal carbonate is present. Add 0.02 N sulfuric acid from a buret until the coloration disappears.

The phenolphthalein alkalinity in p.p.m. of calcium carbonate is equal to the number of cc. of 0.02 N sulfuric acid used multiplied by 20 if 50 cc. of the sample were used, or by 10 if 100 cc. were used. The procedure with cresolphthalein and thymolsulphonphthalein is the same.

(b) *With Methyl Orange*.—Add 2 drops of methyl orange indicator to 50 or 100 cc. of the sample, or to the solution used for the phenolphthalein alkalinity. If the solution becomes yellow, hydroxide, normal carbonate or bicarbonate is present. Add 0.02 N sulfuric acid until the faintest pink coloration appears. This end point is reached when the color of the solution is no longer pure yellow. A good procedure is to titrate a blank of 50 or 100 cc. of distilled water containing 2 drops of methyl orange to a readily recognizable pink, noting the cc. of acid required. All samples are then titrated to this color and the cc. of acid used in the blank deducted from the result.

The methyl orange alkalinity in p.p.m. of calcium carbonate is equal to the number of cc. of 0.02 N sulfuric acid used multiplied by 20 if 50 cc. of the sample were used. The procedure with methyl orange.

(c) *With Erythrosine*.—When methyl orange is objectionable, measure 100 cc. of the sample into a 250-cc. glass-stoppered bottle of colorless glass,

add 2.5 cc. of erythrosine indicator and 5 cc. of chloroform which is neutral to erythrosine. Run in the 0.02 N acid a few drops at a time, shaking the bottle vigorously between each addition. The rose color should slowly disappear until a white paper held back of the bottle fails to reveal a trace of pink in the liquid above the chloroform.

The calculation is the same as that for methyl orange.

3. **Expression of Results.**—Results are to be expressed as phenolphthalein alkalinity, methyl orange alkalinity, etc., in p.p.m. of calcium carbonate.

$$\text{P p m alkalinity as CaCO}_3 = \text{Cc. 0.02 N acid used} \times \frac{1000}{\text{Volume of sample, in cc.}}$$

The alkalinity may be calculated in terms of the amounts of bicarbonate, normal carbonate, and hydroxide, expressed as calcium carbonate, from the figures given in Table 28.

TABLE 28.—RELATIONS BETWEEN ALKALINITY TO PHENOLPHTHALEIN AND THAT TO METHYL ORANGE IN THE PRESENCE OF HYDROXIDE, CARBONATE AND BICARBONATE

Result of titration	Kind of alkalinity as CaCO ₃		
	Hydroxide	Carbonate	Bicarbonate
P = O	0	0	T
P < ½T	0	2P	T-2P
P = ½T	0	2P	0
P > ½T	2P-T	2(T-P)	0
P = T	T	0	0

T = Total alkalinity to methyl orange or similar indicator.

P = Alkalinity to phenolphthalein or similar indicator.

However, values derived from this table are likely to be quite inaccurate, especially when the total alkalinity is less than 100 p.p.m. The accurate calculation of the kinds and amounts of alkalinity present is very complicated and is unnecessary for most purposes. For directions, see Public Health Reports for January 15, 1943, or Journal of the American Water Works Association, Vol. 31, p. 51, 1939.

XI. Acidity.—Waters may have an acid reaction because of the presence of free carbon dioxide, mineral acids or some of their salts, and especially the salts of iron and aluminum which hydrolyze to free hydrogen ion. It is determined by titration with a standard solution of a strong alkali to certain arbitrary standard points or hydrogen ion concentrations. Indicators are selected which show definite color changes at these points.

Calculation of free carbon dioxide, mineral acids and sulfates of iron and aluminum from titration results is often very difficult. Definite rules, covering all cases, cannot be given.

1. **Reagents.**—(a) *Sodium Hydroxide*, N/44 or N/50.—Prepare a saturated solution of sodium hydroxide, and allow to stand in a stoppered pyrex flask until the sodium carbonate settles out. The supernatant liquid will be about 20 N. carbon dioxide-free wa benzoic acid dissolved alcohol, or against weighed portions of acid potassium phthalate, or against the 0.02 N sulfuric acid used in the alkalinity determination, if this is considered sufficiently reliable. Conduct all titrations in such a manner as to prevent exposure of the sodium hydroxide solution to the carbon dioxide of the air.

Adjust the solution to the exact strength desired and preserve in containers of resistant glass, protected from the air by soda-lime tubes. Restandardize at least once in two weeks. Either N/44 or N/50 sodium hydroxide may be used for all acidity determinations provided that the results are correctly calculated.

(b) *Phenolphthalein Indicator*.—(c) *Methyl Orange Indicator*.—These are the same as used for Alkalinity.

olphthalein
n evaporat-
um hydrox-

acidity in terms of p.p.m. of calcium carbonate is equal to the number of cc. of sodium hydroxide solution used multiplied by 20 if 50 cc. were used or by 10 if 100 cc. were used. In the presence of aluminum sulfate and certain other salts of this nature, this determination is of little value as its true end point is reached with extreme slowness or not at all.

(b) *Free Carbon Dioxide*.—Pour 100 cc. of the sample into a tall narrow vessel, preferably a Nessler tube. Add 10 drops of phenolphthalein indicator and titrate rapidly with the N/44 sodium hydroxide solution, stirring gently until a faint but permanent pink color is produced. The free carbon dioxide in p.p.m. is equal to ten times the number of cc. of sodium hydroxide used. In terms of p.p.m. of calcium carbonate it is equal to 2.272 times this figure. Because of the ease with which free carbon dioxide escapes from water, particularly when present in large amounts, a special sample should be collected for this determination and the determination should be made at the time and place of collection. When this is impracticable approximate results may be obtained with a water which does not contain too great a quantity of carbon dioxide by catching samples for this titration in bottles completely filled with the specimen. Such bottled samples should be kept at temperatures below that at the time of collection until ready for examination. If mineral acids or certain salts are present appropriate corrections must be made. At best the results of this titration are uncertain because the proper end point for correct results differs in color with different types of water. The free CO_2 may be calculated from the pH value and the alkalinity by the formula:

$$\text{CO}_2 = 9.70 \times 10^{13} (\text{H}^+) \times \frac{\frac{\text{alk.}}{50,000} + (\text{H}^+) - \frac{10^{-14}}{(\text{H}^+)}}{1 + \frac{11.22 \times 10^{-11}}{(\text{H}^+)}}$$

in which CO_2 = p.p.m. of carbon dioxide as CO_2

alk = p.p.m. total alkalinity as CaCO_3

(H^+) = hydrogen ion concentration, moles per liter, obtained from

$$\text{the formula } \text{pH} = \log \frac{1}{(\text{H}^+)}$$

(c) *Free Mineral Acids*.—Add 2 drops of methyl orange indicator to 50 or 100 cc. of the sample and titrate with 0.02 N sodium hydroxide until the pink coloration disappears. The acidity due to free mineral acids, expressed in terms of calcium carbonate, is equal to the number of cc. of sodium hydroxide used multiplied by 20 if 50 cc. of the sample were used or by 10 if 100 cc. were used.

If appreciable amounts of ferric and aluminum salts are present the results of this procedure are too high. In such cases more accurate estimations can be made by subtracting the acidity due to these salts, calculated from the determined amounts of these substances, from the acidity due to free mineral acids and sulfates of iron and aluminum as determined in the following procedure.

(d) *Mineral Acids and Sulfates of Iron and Aluminum.*—Modify the procedure given above by titrating the sample at boiling temperature in the presence of phenolphthalein indicator. The calculation of results expressed in terms of calcium carbonate, is the same as above.

XII. Hydrogen Ion Concentration; pH Value.—The hydrogen ion concentration is a measure of the intensity of the acid or alkaline properties of a water; the foregoing determinations of alkalinity and acidity measure the quantity of these properties.

The hydrogen ion concentration may be expressed as moles of ionized hydrogen per liter, but it is more usual and convenient to use the pH value, which is the logarithm of the reciprocal of the hydrogen ion concentration.

The pH value may be determined colorimetrically or electrometrically, the former method being best adapted for small laboratories and for field work, and the latter more accurate but requiring more delicate and expensive equipment.

A. Colorimetric Method.—This method involves the addition to the sample of an appropriate indicator, the color of which varies with the pH value. The color obtained is matched either against a set of buffer solutions of known pH value containing the indicator, or glass discs colored to correspond to the appropriate scale of pH values. The standards and reagents for this test are commonly furnished ready for use, but may be made up in the laboratory in accordance with the directions given in Standard Methods. The pH value may be determined to the nearest 0.1 unit by the colorimetric method.

B. Electrometric Method.—This involves placing the sample in contact with an electrode system the voltage of which varies with the pH of the medium, and measuring the voltage developed by means of an accurate potentiometer. The electrode systems used include the hydrogen, antimony, quinhydrone, and glass electrodes, the latter being the most suitable for general laboratory use. The equipment is usually purchased as a unit, and detailed directions for its use are provided. The electrometric method is the basic method of pH determination, and standards for the colorimetric method should be checked electrometrically.

XIII. Chloride.—Chloride in water may be derived from mineral deposits, from ocean vapors carried inland by the wind, or from polluting materials like sewage and industrial wastes.

1. Reagents.—(a) *Standard Sodium Chloride Solution.*—Dissolve 16.49 gm. of pure fused sodium chloride in distilled water and make up to 1 liter. Dilute 100 cc. of this stock solution to 1 liter. One cc. contains 1 mg. of chloride radicle.

(b) *Standard Silver Nitrate Solution.*—Dissolve about 2.40 gm. of silver nitrate crystals in 1 liter of distilled water. Titrate against 3 to 10 cc. of the standard salt solution diluted to 50 cc. with distilled water, using the potassium chromate indicator as directed in the procedure. Correct for

the error due to variations in the volume of the liquid by means of the formula

$$X = 0.003V + 0.02$$

in which X is the correction in cc. to be deducted from the volume of silver nitrate used, and V equals the total cc. of liquid in the mixture at the end so that 1 cc. is exactly equivalent to

—Dissolve 50 gm. of neutral potassium permanganate in 1 liter of distilled water. Add silver nitrate to produce a slight red precipitate, allow to stand a day or two, filter and dilute the filtrate to 1 liter with distilled water.

(d) *Aluminum Hydroxide*.—Prepare as directed on page 258.

2. *Procedure*.—(a) *Preparation of the Sample*.—If the sample has a color greater than 30, decolorize by shaking it thoroughly with washed aluminum hydroxide, 3 cc. to 500 cc. of the sample, and allow the precipitate to settle. Make the determination on an aliquot portion of the clarified sample, filtered if necessary.

Samples which are acid should be neutralized with sodium carbonate. If hydroxide is present neutralize with dilute sulfuric acid until the color of the phenolphthalein indicator used is discharged in the cold. If the sample contains hydrogen sulfide, acidify with sulfuric acid, boil a few minutes, cool, neutralize with NaHCO_3 , and restore the original volume by adding distilled water.

With waters high in chloride, use 25 cc. or even a smaller amount, diluting the volume taken to 50 cc. with distilled water. If the amount of chloride is very low, add a definite amount of standard chloride solution before titration, or concentrate 250 cc. of the sample to 50 cc. by evaporation. Rotate the liquid to make sure that no residue remains undissolved on the walls of the dish, assisting in this operation by using a rubber-tipped glass rod.

(b) *Titration*.—Add 1 cc. of potassium chromate indicator to 50 cc. of the prepared sample in a 150-mm. white porcelain evaporating dish or a 150-cc. Erlenmeyer flask over a white surface. Titrate with the silver

comparison of the color in the titrating vessel with that in a similar vessel containing the same quantity of indicator in 50 cc. of distilled water. Some analysts in a dark room provided with a yellow light by daylight, the end point is very distinct by using a photographic glass. Plain Mazda lamps coated with several coats of "yellow dipping liquid" are excellent; most convenient, probably, is the use of dark amber goggles.

3. *Calculation*.—Correct for volume as directed above.

$$\text{P.p.m. chloride} = 500 \times \frac{\text{Corrected cc. silver nitrate solution}}{\text{Volume of sample in cc.}}$$

coloring matter, and chlorates are examples of interfering substances,

which must be considered in evaluating results. As in all colorimetric determinations, turbidity interferes.

The wide use of chlorine in the treatment of water has created the need for a reliable test for the presence of free chlorine. The most widely used indicator is ortho-tolidine, which must not be confused with ortho-toluidine which is an entirely different chemical compound.

1. **Reagents.**—(a) *Ortho-tolidine Solution.*—Weigh out 0.5 gm. of ortho-tolidine, and grind to a thin paste in a mortar with 5 cc. of dilute hydrochloric acid, previously made by adding 150 cc. of concentrated hydrochloric acid (sp. gr. 1.18 to 1.19) to 350 cc. of distilled water. Add to the paste 150 to 200 cc. of distilled water. The ortho-tolidine should go into solution immediately. Transfer to a 1000-cc. graduate, and make up to 505 cc. with distilled water. Add the balance of the dilute hydrochloric acid. This should make the final volume 1000 cc.

(b) *Sodium Hydroxide Solution*, 4.5 N.

(c) *Magnesium Sulfate Solution.*—Dissolve 20 gm. of magnesium sulfate in 100 cc. of distilled water. If necessary, eliminate any large chlorine demand in this and the preceding solution by treating to a slight residual with calcium or sodium hypochlorite solution.

(d) *Potassium Chromate-Dichromate Solutions.*—(1) Weak.—Dissolve 0.25 gm. of potassium dichromate and 0.75 gm. of potassium chromate in 1 liter of distilled water. (2) Strong.—Dissolve the same quantities of potassium dichromate and chromate in 100 cc. of distilled water.

(e) *Boric Acid-Borax Buffer*, pH 6.5.—Dissolve 12.4 gm. of boric acid in distilled water and make up to 1 liter. This solution is approximately 0.2 M and has a pH of about 4.6. Dissolve 3.8 gm. of borax ($\text{Na}_2\text{B}_4\text{O}_7$) in distilled water and make up to 1 liter. This is 0.01 M and has a pH of about 9.2. Add sufficient of the borax solution, usually about 80 cc. to 1 liter of the boric acid solution to produce a buffer solution of pH 6.5.

2. **Procedure.**—Place 100 cc. of sample in a 100-cc. Nessler tube, and warm to 20° C., if necessary. Add 2 cc. of ortho-tolidine solution, mix, and place in the dark for not less than five or more than fifteen minutes.

Small amounts of chlorine give a yellow, and larger amounts an orange color. For quantitative estimations, compare the color with that of the permanent standards given below. All tubes should match as to height of graduation mark.

Interference by nitrites up to 1 p.p.m. is avoided by this procedure.

3. **Permanent Standards.**—Place in matched 100-cc. Nessler tubes, the volumes of dichromate-chromate solutions given in Table 29, and make up to 100-cc. mark with the boric acid-borax buffer.

TABLE 29—CHLORINE STANDARDS, SCOTT FORMULA

Chlorine, p.p.m.	Cc. weak dichromate- chromate	Chlorine, p.p.m.	Cc. strong dichromate- chromate
0 01	0 4	0 50	2 6
0 02	0 8	0 70	3 6
0 03	1 3	1 00	5 2
0 05	2 2	1 50	8 0
0 07	3 1	2 00	11 2
0 10	4 4	3.00	18 0
0 15	7 4		
0 20	10 0		
0 25	13 0		
0 30	16 0		
0 40	21 0		

If the water contains over 1 p.p.m. of iron or 0.01 p.p.m. of manganic manganese, flocculate 200 cc. with 2 cc. of the magnesium sulfate solution and 2 cc. of 4.5 N sodium hydroxide, clarify by centrifugalizing, and follow the standard procedure.

XV. Dissolved Oxygen.—Dissolved oxygen is determined by the Rideal-Stewart modification of the Winkler method on all polluted waters and those containing 0.1 p.p.m. or more of nitrite nitrogen. On other waters the original Winkler method should be used. As little as 1 p.p.m. of ferrous

Winkler procedure the first three steps are omitted. The procedure outlined should be followed from the addition of manganous sulfate except that the volume of alkaline potassium iodide added should be reduced to 1 cc.

1. Reagents.—(a) *Sulfuric Acid, Concentrated.*—Specific gravity, 1.83 to 1.84.

(b) *Potassium Permanganate.*—Dissolve 6.32 gm. of the salt in distilled water and dilute the solution to 1 liter.

(c) *Potassium Oxalate* ($K_2C_2O_4 \cdot H_2O$).—Dissolve 20 gm. in distilled water and dilute the solution to 1 liter.

(d) *Manganous Sulfate* ($MnSO_4 \cdot 4H_2O$).—Dissolve 480 gm. in water and dilute to 1 liter.

(e) *Alkaline Potassium Iodide.*—Dissolve 700 gm. of potassium hydroxide or 500 gm. of sodium hydroxide and 150 gm. of potassium iodide in water and dilute the solution to 1 liter.

(f) *Sodium Thiosulfate, 0.025 N*.—Dissolve 2.48 gm. of chemically pure recrystallized sodium thiosulfate in distilled water and dilute the solution to 1 liter with distilled water. Each cc. is equivalent to 0.2 mg. of oxygen or to 0.1395 cc of oxygen at 0° C. and 760 mm. of pressure. Since this solution is not permanent it should be standardized occasionally against a 0.025 N solution of potassium dichromate (1.225 gm. per liter).

For the standardization about 5 gm. of potassium iodide are dissolved in about 50 cc. of distilled water in a glass-stoppered bottle, and 10 cc. of dilute sulfuric acid (1 to 10) are added, followed by 40 cc. of standard dichromate solution. The mixture is placed in the dark for five minutes, then diluted to about 400 cc. and titrated with the sodium thiosulfate in the same manner as in the dissolved oxygen procedure.

(g) *Starch Solution.*—Grind 5 gm. of potato starch in a mortar with enough cold water to make a paste. Pour into 1 liter of boiling distilled water, stir, and allow to settle overnight. Use the clear supernatant, preserving, if desired, with 1.25 gm. of salicylic acid per liter.

2. Collection of Sample.—Collect the sample in a narrow-necked glass-stoppered bottle of 250 to 270 cc. capacity. The absorption or entrainment of atmospheric oxygen is avoided by filling the bottle through a glass or rubber tube extending well into the tap from which the water is taken and to the bottom of the bottle. To avoid air bubbles allow the bottle to overflow for several minutes, and then replace the glass stopper carefully so that no air bubble is left. In collecting from a pond or tank connect the sample bottle to a bottle of 1 liter capacity. Provide each bottle with a

2-hole rubber stopper having one glass tube extending to the bottom and another tube entering but not projecting into the bottle. Connect the short tube of the sample bottle to the long tube of the liter bottle. Immerse

of the sample bottle. Lower the two bottles in a weighted cage to the desired depth. Water entering during the descent will be flushed through into the liter bottle. When air bubbles cease rising to the surface raise the bottle and replace the perforated stopper with a solid one in such a manner as to avoid entraining bubbles of air.

3. Procedure.—Remove the stopper from the sample bottle and add first 0.7 cc. of concentrated sulfuric acid and then 1 cc. of the potassium permanganate solution. These and all other reagents should be introduced by pipet under the surface of the liquid. Insert the stopper and mix by inverting the bottle several times. If a noticeable excess of potassium permanganate is not present at the end of twenty minutes again add 1 cc. of the permanganate solution; if this is still insufficient, use a stronger permanganate solution. After twenty minutes destroy the excess of permanganate by adding 1 cc. of potassium oxalate solution, restopper the bottle at once and mix its contents. Add 1 cc. of the manganous sulfate solution and 3 cc. of the alkaline potassium iodide solution. Allow the precipitate to settle, then add 1 cc. of concentrated sulfuric acid and mix by shaking.

The procedure to this point must be carried out at the point of collection, but after the acid has been added and the stopper replaced there is no further change, and the rest of the test may be performed within a few hours, as convenient.

Transfer 200 cc. of the contents of the bottle to a flask and titrate with 0.025 N sodium thiosulfate, using a few cc. of the starch solution as an indicator toward the end of the titration. Do not add the starch solution until the color has become faint yellow. Titrate until the blue color disappears.

4. Calculation of Results.—Oxygen is reported in p.p.m. by weight. It is sometimes convenient to know the number of cc. per liter, of the gas at

sample are taken, the number of cc. of 0.025 N thiosulfate used is equal to p.p.m. of oxygen. Corrections for volume of reagents added amount to less than 3 per cent and are not necessary except in work of unusual precision. To obtain the result in cc. per liter multiply the number of cc. of thiosulfate used by 0.698. To obtain the result in percentage of saturation, divide the p.p.m. of dissolved oxygen found by the figure given in Table 30, for the appropriate temperature and chloride content.

The last column of the table permits interpolation for intermediate chloride values. At elevations differing considerably from mean sea level attention must be given to barometric pressure, the normal pressure in the region being preferable to the specific pressure at the time of sampling. The term "saturation" refers to a condition of equilibrium between the solution and an oxygen pressure in the atmosphere corresponding to 158.8

mm. or approximately one-fifth atmosphere. The true saturation or equilibrium between the solution and pure oxygen is nearly five times this value, and consequently values in excess of 100 per cent saturation frequently occur in the presence of oxygen-forming plants.

TABLE 30.—SOLUBILITY OF OXYGEN IN FRESH WATER AND IN SEA WATER OF STATED DEGREES OF SALINITY AT VARIOUS TEMPERATURES WHEN EXPOSED TO AN ATMOSPHERE CONTAINING 20.9 PER CENT OF OXYGEN UNDER A PRESSURE OF 760 MM.*

Temperature ° C	Chloride in sea water (p p m)					Difference per 100 p p m chloride
	0	5000	10,000	15 000	20 000	
	Dissolved oxygen in parts per million					p p m
0	14.62	13.79	12.97	12.14	11.32	0.0165
1	14.23	13.41	12.61	11.82	11.03	0.0160
2	13.84	13.05	12.28	11.52	10.76	0.0154
3	13.48	12.72	11.98	11.24	10.50	0.0149
4	13.13	12.41	11.69	10.97	10.25	0.0144
5	12.80	12.09	11.39	10.70	10.01	0.0140
6	12.48	11.79	11.12	10.45	9.78	0.0135
7	12.17	11.51	10.85	10.21	9.57	0.0130
8	11.87	11.24	10.61	9.98	9.36	0.0125
9	11.59	10.97	10.36	9.76	9.17	0.0121
10	11.33	10.73	10.13	9.55	8.98	0.0118
11	11.08	10.49	9.92	9.35	8.80	0.0114
12	10.83	10.28	9.72	9.17	8.62	0.0110
13	10.60	10.05	9.52	8.98	8.46	0.0107
14	10.37	9.85	9.32	8.80	8.30	0.0104
15	10.15	9.65	9.14	8.63	8.14	0.0100
16	9.95	9.46	8.96	8.47	7.99	0.0098
17	9.74	9.26	8.78	8.30	7.84	0.0095
18	9.54	9.07	8.62	8.15	7.70	0.0092
19	9.35	8.89	8.45	8.00	7.56	0.0089
20	9.17	8.73	8.30	7.86	7.42	0.0088
21	8.99	8.57	8.14	7.71	7.28	0.0086
22	8.83	8.42	7.99	7.57	7.14	0.0084
23	8.68	8.27	7.85	7.43	7.00	0.0083
24	8.53	8.12	7.71	7.30	6.87	0.0083
25	8.38	7.96	7.56	7.15	6.74	0.0082
26	8.22	7.81	7.42	7.02	6.61	0.0080
27	8.07	7.67	7.28	6.88	6.49	0.0079
28	7.92	7.53	7.14	6.75	6.37	0.0078
29	7.77	7.39	7.00	6.62	6.25	0.0076
30	7.63	7.25	6.86	6.49	6.13	0.0075

* The solubility, B, under any other barometric pressure can be obtained from the corresponding value in the table by the formula:

$$S' = S \frac{B}{760} = S \frac{B'}{29.92}$$
 in which S' = solubility at B or B' , S = solubility at 760 mm. or 29.92 in., B = barometric pressure in mm., and B' = pressure in inches.

MINERAL ANALYSIS OF WATER

The procedures outlined in the following paragraphs are designed for the examination of potable waters or those to be rendered potable by purification. The elements or radicles determined are to be reported as

such in parts per million and in milli-equivalents. Milli-equivalents are calculated from the amount of any given ion in parts per million by dividing that value by the combining weight (element or radicle weight divided by the valence) of that element or radicle.

Procedures for the determination of manganese, copper, lead, tin and zinc have not been given. If their determination is desired, refer to the latest edition of Standard Methods of Water Analysis.

Dissolved solids, alkalinity and acidity, chlorides and nitrates are determined as outlined in the corresponding sections in Chemical Examination.

I. Silica.—A. Gravimetric Method.—Evaporate in a platinum dish 100 to 1000 cc. of the sample, or sufficient, if possible, to form a residue weighing 0.4 to 0.6 gm. and preferably containing 0.1 to 0.2 gm. of calcium. When the residue is nearly dry add 1 cc. of hydrochloric acid (1 to 1) and evaporate to dryness; if much organic matter is present char it in a radiator. Moisten the residue with dilute hydrochloric acid and expel the excess of acid by heating on a water bath. Add a few drops of hydrochloric acid, dissolve in with hot water. Evaporate the and combine the two residues.

Id 2 drops of concentrated sulfuric acid and a little hydrofluoric acid, volatilize the acids, ignite and weigh again. Report the loss in weight as silica (SiO_2).

B. Colorimetric Method.—1. Reagent.—(a) *Ammonium Molybdate Solution*.—Dissolve 30 gm. of ammonium molybdate in 200 cc. of 1 to 1 hydrochloric acid and dilute to 600 cc. with distilled water.

2. Standards.—

water. One cc. o

standards may be
when 50 cc. of sample are used.

3. Procedure.—Measure 50 cc. of the sample into a tall form Nessler tube and add 5 cc. of the molybdate reagent. Thoroughly shake the mixture and allow it to stand about fifteen minutes but not longer than twenty minutes. The yellow color developed is then compared with the chromate standards.

TABLE 31 —COLOR STANDARDS FOR THE DETERMINATION OF SILICA

Cc. of chromate diluted to 55 cc	P p m SiO_2 when 50 cc sample used
0 0	0
0.1	2
0.2	4
0.3	6
0.4	8
0.5	10
0.6	12
0.7	14
0.8	16
0.9	18
1 0	20
1 1	22
1.2	24
1.3	26
1.4	28
1.5	30

In using this method phosphates interfere and strong mineral acids in excess of that present in the reagent will prevent the development of

maximum color. The method will not determine suspended silica. Hydrogen sulfide gives a blue color instead of yellow and must, therefore, be removed.

II. Iron.—Iron may be present in natural waters and in those receiving industrial waste in the ferric or ferrous condition and as soluble, colloidal or insoluble compounds. The ferric form is rarely found in solution in appreciable quantities except in acid waters. Insoluble or colloidal iron is usually all ferric. Ferrous iron is readily oxidized to the ferric condition and most if not all of the iron present in a water will be in the ferric condition by the time the sample reaches the laboratory. Determinations of ferrous iron should, therefore, be made at the source of supply.

A. Total Iron, Colorimetric Method.—1. **Reagents.**—(a) *Standard Ferric Solution.*—Dissolve 0.7022 gm. of crystallized ferrous ammonium sulfate in 50 cc. of distilled water and 20 cc. of concentrated sulfuric acid. Warm the solution and add potassium permanganate until the iron is completely oxidized. Dilute to 1 liter. One cc. contains 0.1 mg. of iron.

(b) *Potassium (or Ammonium) Thiocyanate.*—Dissolve 2 gm. in distilled water and make up to 100 cc.

(c) *Hydrochloric Acid, Dilute.*—One volume of concentrated acid (specific gravity 1.2) to 3 volumes of distilled water. Approximately 3 N.

(d) *Potassium Permanganate.*—Approximately 0.2 N. Dissolve 6.30 gm. in distilled water and make up to 1 liter.

(e) *Hydrochloric Acid, Concentrated*—Free from iron.

(f) *Nitric Acid, Concentrated*—Specific gravity 1.42; free from iron.

(g) *Nitric Acid, Dilute*—Specific gravity 1.195; 352 cc. of concentrated acid (specific gravity 1.42) in 1 liter. Approximately 6.0 N.

2. **Procedure.**—Evaporate 100 cc. or less of the sample to dryness or use the solids from the determination of residue on evaporation. With silt-bearing waters the quantity of iron may be so great that as little as 10 cc. of the sample will be sufficient. With such waters 5 to 10 cc. of concentrated hydrochloric acid should be added prior to evaporation. If much organic matter is present destroy it by ignition, being careful not to prolong ignition to such an extent as to render the iron nearly insoluble. Heat to drive off the excess acid if any was used, provided ignition was not done. Cool and add 0.8 to 1 cc. of 3 N hydrochloric acid. Warm on the water bath avoiding evaporation to dryness by adding small quantities of distilled water. Rinse the hot solution into a 50-cc. Nessler tube, filtering if necessary. Add 1 or 2 drops of potassium permanganate solution; if the color of the permanganate does not persist for at least five minutes, add more, drop by drop. Cool and dilute to the mark with distilled water. With the iron standards prepared and ready, add 5 cc. of thiocyanate solution to the sample and to the standards, mix and compare immediately. With permanent standards comparison must immediately follow the addition of thiocyanate.

If the sample is low in organic matter, boil 50 cc. of the sample with 5 cc. of 6 N nitric acid for five minutes, add 3 drops of permanganate solution and cool. Add 5 cc. of thiocyanate and compare immediately with standards made from the standard iron solution, using 5 cc. of 6 N nitric acid instead of the hydrochloric acid.

3. **Preparation of Iron Standards.**—Measure into matched Nessler tubes volumes of standard iron solution from 0.5 to 4 cc., covering the range of standards required for the usual determination. Dilute to about 40 cc.,

and titrate the excess of iodine with thiosulfate solution in the presence of starch indicator.

3. **Calculation.**—The difference in the number of cc. of thiosulfate solution used in the titration of the standard iodine solution and in the back titration of the sample multiplied by 0.3408 is equal to the p.p.m. of hydrogen sulfide, free and combined. This method does not give satisfactory results in sewage analysis and should not be used for this purpose.

IV. **Sulfates.**—A qualitative test may be made by adding 1 cc. of concentrated hydrochloric acid to 100 cc. of clear sample. Warm and add a little barium chloride solution; a white precipitate of barium sulfate is produced, which is insoluble in hydrochloric acid.

1. **Reagents.**—(a) *Hydrochloric Acid, Concentrated.*—Specific gravity 1.2.

(b) *Hydrochloric Acid, Dilute.*—1 to 1.

(c) *Sulfuric Acid, Concentrated.*—Specific gravity 1.84.

(d) *Barium Chloride Solution, 10 per cent.*

2. **Procedure.**—Evaporate a volume of sample sufficient to give 0.4 to 0.6 gm. of residue in a platinum dish; usually 100 cc. are sufficient but a larger volume may be required. When almost dry add 1 cc. of dilute hydrochloric acid and continue the evaporation to dryness. If much organic matter is present char the residue in a muffle furnace. Moisten the residue with dilute hydrochloric acid and take down to dryness on a water bath. Ignite and weigh the insoluble precipitate. Add 2 drops of sulfuric acid and a few drops of hydrofluoric acid, heat to volatilize the acids, ignite and weigh; the loss in weight is silica. Add 2 cc. of hydrochloric acid bringing it into contact with all of the residue; add about 20 cc. of water and boil, filter if necessary, thoroughly washing the dish and filter with hot water. Add a slight excess of hot barium chloride solution and warm, stirring at intervals for one-half hour until the precipitate settles readily and the supernatant liquid becomes clear. Filter, wash the residue with hot water, dry, ignite, and weigh.

3. **Calculation.**—The weight of the residue in mg. multiplied by 100 gives the p.p.m. of sulfate as SO_4 .

V. **Calcium.**—1. **Reagents.**—(a) *Nitric Acid, Concentrated.*

(b) *Ammonium Hydroxide.*

(c) *Ammonium Oxalate, saturated solution.*

(d) *Hydrochloric Acid, Dilute.*

2. **Procedure.**—Heat to boiling the filtrate from the silica determination, page 272, oxidize with concentrated nitric acid, and concentrate to about 100 cc. Add ammonium hydroxide in slight excess, boil for a minute and filter. Add to the filtrate an excess of fresh saturated solution of ammonium oxalate, little by little. The solution must be kept warm and stirred at intervals until the precipitate settles readily and leaves a clear supernatant liquid. Filter, dissolve the precipitate in a little hot dilute hydrochloric acid, and reprecipitate with ammonium hydroxide and ammonium oxalate. If great accuracy is not required the solution and reprecipitation may be omitted, and the first precipitate washed clean with hot water. Save the filtrate for the determination of magnesium. Ignite the precipitate and weigh it as calcium oxide, 71.46 per cent of which is the equivalent of calcium; or dissolve the precipitate in hot 2 per cent sulfuric acid and titrate with a standard solution of potassium permanganate.

VI. Magnesium.—1. Reagents.—(a) *Sodium Ammonium Phosphate*, saturated solution.

(b) *Hydrochloric Acid, Concentrated*.

(c) *Hydrochloric Acid, Dilute*.

(d) *Ammonium Hydroxide*, 3 per cent.

2. Procedure.—Acidify the filtrate from V with hydrochloric acid and concentrate it to about 100 cc. Add 20 cc. of the solution of sodium ammonium phosphate, cool, and make slightly alkaline by adding ammonium hydroxide drop by drop. Allow to stand four hours, then filter and wash the precipitate with 3 per cent ammonium hydroxide. Dissolve the precipitate, especially in the presence of large amounts of sodium or potassium, in a slight excess of dilute hydrochloric acid and reprecipitate the magnesium with ammonium hydroxide and a few drops of the sodium ammonium phosphate solution. This solution and reprecipitation may be omitted if great accuracy is not required. Ignite the precipitate and weigh it as magnesium pyrophosphate, 21.84 per cent of which is the equivalent of the magnesium. If manganese is present it is precipitated with the magnesium and a correction for it must be applied after determining manganese in a separate sample.

VII. Sodium and Potassium.—1. Reagents.—(a) *Barium Hydroxide*, saturated solution.

(b) *Ammonium Hydroxide*.

(c) *Ammonium Carbonate Solution*.

2. Procedure.—Evaporate the filtrate after the removal of silica and sulfates in IV to dryness. Heat the residue barely to redness to remove any ammonium salts. Dissolve in 25 to 100 cc. of water, add an excess of saturated barium hydroxide solution and heat to boiling. Allow to stand

To the

heat on

... .. is a clear supernatant. Filter and evaporate the filtrate to dryness; ignite to expel any ammonium salts. Take up the residue in a few cc. of hot water, filter, and wash, keeping the volume small. Repeat the addition of ammonium hydroxide and ammonium carbonate and the succeeding operations until no precipitate is formed on the addition of these reagents. Transfer the final filtrate to a small platinum dish, add a few drops of hydrochloric acid and evaporate to dryness. Heat gently to drive off ammonium salts and finally heat barely to redness. Cool and weigh. Take up the sodium and potassium chlorides in a few cc. of water, filter through a small paper and wash. Ignite the filter paper in the platinum dish, cool and weigh. The

reference should be made to the latest edition of Standard Methods of Water Analysis.

PHYSICAL AND CHEMICAL CHARACTERISTICS OF ACCEPTABLE WATERS

Water to be suitable for drinking and culinary purposes should be clear, colorless, odorless and pleasant to the taste; it should be free from toxic salts, and should not contain excessive amounts of soluble mineral substances, nor of any chemical used in treatment. The quality standards in

this section are those given by the U. S. Public Health Service in Public Health Reports for January 15, 1943.

I. Physical Characteristics.—1. **Turbidity.**—Turbidity should not exceed 10 p.p.m. or, preferably, 5 p.p.m. (silica scale). 2. **Color.**—Color should not exceed 20 p.p.m. or, preferably, 10 p.p.m. on the standard platinum cobalt scale. 3. **Odor and Taste.**—The water should be substantially free from odor and taste due to hydrogen sulfide, chlorine, phenol and other chemicals, and from odor due to the presence of microscopic organisms.

II. Chemical Characteristics.—The presence of the following substances constitutes grounds for condemnation of a supply:

Lead in excess of 0.1 p.p.m. as Pb

Fluoride in excess of 1 p.p.m. as F

Arsenic in excess of 0.05 p.p.m. as As

Selenium in excess of 0.05 p.p.m. as Se

Salts of barium, hexavalent chromium, heavy metal glucosides and other substances known to have deleterious physiological effects should not be permitted in the water supply system. If the water supply is a temporary one, to be consumed by adults only, fluoride concentrations several times that given above may be permitted.

The following substances should preferably not occur in excess of the concentrations in parts per million given below:

Copper	3 0
Iron and manganese (total)	0 3
Magnesium	125 0
Zinc	15 0
Chloride	250 0
Sulfate	250 0
Phenolic compounds as phenol	0 001
Total solids	1000 0

For treated waters, the following additional requirements are pertinent:

1. The pH value should not exceed 10.6 at 25° C.
2. The normal carbonate alkalinity should not exceed 120 parts per million. This requirement may be met by keeping the total alkalinity within the limits given below for the corresponding pH values.

pH value	Total alkalinity as CaCO ₃
8 to 9.6	400 p.p.m.
9.8	300 p.p.m.
10.0	230 p.p.m.
10.2	190 p.p.m.
10.4	170 p.p.m.
10.6	160 p.p.m.

Temporary supplies to be used by troops for periods not exceeding one or two weeks need not be held too rigidly to the standards given, except those for the definitely toxic substances listed in the first table. A moderate excess of some chemical substance over the limit indicated is less important under such circumstances than good appearance and palatability.

SEWAGE, EFFLUENTS, INDUSTRIAL WASTES AND GROSSLY POLLUTED WATERS

I. Collection of Samples.—1. **Representative Samples.**—Sampling procedures for sewages, industrial wastes, and polluted waters should be adapted to the existing conditions to secure truly representative samples. Single catch samples are generally of little value, since the strengths of sewages and wastes may vary greatly from hour to hour.

solution, and distil off the ammonia. Add 100 cc. of sulfuric acid, 1 cc. of copper sulfate, and if necessary 5 gm. of potassium or sodium sulfate. Digest under a hood for twenty to thirty minutes after the digestate has become clear. Cool and add about 250 cc. of ammonia-free water. Make alkaline with sodium hydroxide, using the blue copper precipitate as an indicator and distil into 50 cc. of boric acid solution until about 200 cc. of distillate have been obtained. Bumping may be reduced with zinc or "boiling chips." Add 3 drops of methyl red, and titrate the ammonia with 0.05 N sulfuric acid, matching the end-point with that of a blank containing the same amounts of boric acid and indicator diluted to the same volume with distilled water. A blank should be run on the reagents and corrections made as indicated.

(b) *Direct Nesslerization*.—Digest as directed above. Rinse the cooled digestate into a 250-cc. flask, and dilute to the mark with ammonia-free water. Pipet 50 cc. into a 100-cc. flask or Nessler tube, add sodium hydroxide slowly until alkaline, keeping the flask and contents cool in running water. Dilute to the mark with ammonia-free water, mix and allow to stand for twenty-four hours. Nesslerize an aliquot portion of the clear supernatant. Run a blank test on the reagents used in the entire process, and correct accordingly.

3. Calculation.—

$$\text{P p m. organic nitrogen} = \frac{700 \times \text{cc. of acid}}{\text{Volume of sample, cc.}}$$

VI. Nitrite Nitrogen.—Place in a Nessler tube 0.1 to 10 cc. of sample, dilute to 50 cc., and determine as in the procedure for water. If necessary, the sample may be first clarified with zinc sulfate and sodium hydroxide, as described in the determination of ammonia nitrogen.

VII. Nitrate Nitrogen.—A. *Reduction Method*.—The method is the same as that described for water. In place of distillation, direct nessleriza-

be subtracted from the final result, as nitrites are also reduced.

B. Phenoldisulfonic Acid Method.—A modification of the phenoldisulfonic acid method described for water may also be used. The sample is first clarified with zinc sulfate or copper sulfate and sodium hydroxide if low in color, or with activated carbon (0.5 gm. in 50 cc.) and aluminum hydroxide paste (1 cc. in 50 cc.) if high in color. Chlorides must also be removed if over 30 p.p.m. The clarified sample is evaporated, moistened with 1 cc. of phenoldisulfonic acid, diluted, and made alkaline.

It is then treated with 0.5 gm. of activated carbon, filtered, made up to volume and compared with standards. New supplies of activated carbon should be tested for suitability by treating a 0.01 mg. nitrate standard with 1 gm. of the carbon, filtering, and noting if any change in color takes place. If there is no detectable change the carbon is suitable for use.

VIII. Oxygen Consumed from Permanganate.—Since permanganate attacks different proportions of the total carbon in different sewages and wastes, the oxygen consumed test is not an accurate measure of the carbonaceous organic matter. The test is useful mainly for estimating the

strength of certain trade wastes and sewages, the biochemical oxygen demand of which cannot be determined, and as a rapid measure of the efficiency of purification processes in routine work.

1. **Reagents.**—(a) *Dilute Sulfuric Acid.*—Add 1 volume of C.P. sulfuric acid to 3 volumes of water.

(b) *Ammonium Oxalate Solution.*—Dissolve 0.8880 gm. of C.P. ammonium oxalate in 1 liter of distilled water. One cc. is equivalent to 0.1 mg. of oxygen. A fresh solution should be prepared at least once a month.

(c) *Standard Potassium Permanganate.*—Dissolve 0.4 gm. of C.P. potassium permanganate in 1 liter of distilled water. Add 10 cc. of this solution and 10 cc. of the dilute sulfuric acid to 100 cc. of distilled water in an Erlenmeyer flask, and digest thirty minutes in boiling water. Add 10 cc. of the standard ammonium oxalate solution, and then titrate to a pink coloration with the standard potassium permanganate. This destroys the oxygen consuming capacity of the distilled water. Now add 10 cc. of the oxalate solution and titrate with the potassium permanganate. Adjust the permanganate so that 1 cc. is equivalent to 1 cc. of the oxalate or 0.1 mg. of available oxygen. Preserve in a dark glass bottle.

(d) *Sodium Hydroxide Solution.*—Dissolve 500 gm. of sodium hydroxide in 1000 cc. of distilled water. Settle and decant the clear supernatant liquid.

2. **Procedure.**—Place in a flask a suitable volume of sample, 1 to 100 cc., depending on its strength, and dilute to 100 cc. with distilled water. The quantity of sample used should be such that an excess of not less than 5 cc. of the permanganate remains after digestion. Add 10 cc. of the potassium permanganate and 10 cc. of the dilute sulfuric acid. Digest for thirty minutes in boiling water, keeping the flask submerged below the level of the contents. Remove the flask, add 10 cc. of the ammonium oxalate, and titrate back to a faint pink color with the potassium permanganate. Digest a blank made up of 100 cc. of distilled water, 10 cc. of permanganate and 10 cc. of sulfuric acid, and titrate in a similar manner. Compute the cc. of permanganate used by the distilled water added to dilute the sample, and deduct this value from that obtained for the sample.

$$\text{Oxygen consumed, p.p.m.} = \frac{100 \times \text{cc. of KMnO}_4}{\text{cc. of sample}}$$

Corrections should be applied for oxidizable mineral substances such as nitrites, ferrous iron, and sulfides. Direct titration of the acidified sample with the permanganate in the cold to an end-point permanent for three minutes, will serve the purpose.

For brines or waters high in chloride, substitute 0.5 cc. of sodium hydroxide for the sulfuric acid, and add 5 cc. of sulfuric acid before adding the oxalate after digestion.

IX. Dissolved Oxygen.—It has been found that waste substances likely to be present in sewage and industrial wastes interfere with the Winkler procedure for the determination of dissolved oxygen, and modified procedures have been developed to prevent such interference. The modifications are too numerous to be reproduced in detail in a work of this scope, and the reader is referred to the latest edition of *Standard Methods of Water Analysis*. A tabulation of the interfering substances, together with the corrective procedure, and references to the modified procedure used in each case, are given in Table 51.

TABLE 33.—SUBSTANCES INTERFERING WITH DISSOLVED OXYGEN TEST

Substance	Effect on test	Modification used	Description of modification, reason for use
Nitrite, NO_2^- over 0.1 p.p.m.	High results, oxidizes iodide; recurring end-point $2\text{HNO}_2 + 2\text{HI} = \text{N}_2\text{O}_2 + 2\text{H}_2\text{O} + \text{I}_2$ $2\text{N}_2\text{O}_2 + 2\text{H}_2\text{O} + \text{O}_2 = 4\text{HNO}_2$	Rideal-Stewart	Add H_2SO_4 , KMnO_4 , oxidizes NO_2^- to NO_3^- ; remove excess KMnO_4 with oxalate, proceed as in Winkler test
		Aside	Add NaNa , either as preliminary treatment, with acid, or in alkaline iodide; reduces NO_2^- to N_2
		sulfamic acid	$\text{H}(\text{NH}_2)\text{SO}_3$ reduces NO_2^- to N_2 (S. W. J., 13, 542, 1941)
Organic matter over 1000 p.p.m., as dextrose or peptone	Low results, organic matter oxidized by O_2 at pH 12	Short Winkler	Cut period of alkalization, acidify right after shaking (Ind. Eng. Chem. Anal. Ed. 4, 59, 1932)
Sulfites, thiosulfates, polythionates (sulfite pulp mill wastes)	Low results, reduce O_2	Alkaline hypochlorite	Add alkaline-hypochlorite, then acid-KI, neutralize I_2 with Na_2SO_3 , proceed with Winkler test (Ind. Eng. Chem. Anal. Ed. 4, 59, 1932)
Chlorine or hypochlorites 1 p.p.m. $\text{Cl}_2 = 0.23$ p.p.m. O_2	High results, liberating I_2	Partial alkaline hypochlorite	Begin with addition of acid-KI, as above
<p style="text-align: center;">.....</p>			
Ferrous salts 1 p.p.m. $\text{Fe} = 0.14$ p.p.m. O_2	Low results, reduce O_2	Rideal-Stewart with fluoride	KMnO_4 oxidizes Fe^{++} (Ind. Eng. Chem., 15, 1156, 1923)
Suspended organic solids (mud suspensions)	Low results	Alum flocculation	Flocculate with alum and NH_4OH , settle, decant, use short Winkler method (Ind. Eng. Chem. Anal., Ed. 12, 711, 1940).

For sewages uncontaminated by industrial wastes, either the Rideal-

Stewart, and are recommended if the chemicals are obtainable. When industrial wastes are present, the analyst must decide which modification to use on the basis of the kind and amount of interfering substances present, and the degree of accuracy desired.

X. Relative Stability.—The use of this determination is rapidly decreasing in favor of the more significant biochemical oxygen demand test. It is, however, of value in field work and in small laboratories where a simple approximate measure of putrefiable organic matter in relation to oxygen are test

1. Reagents.—(a) *Methylene Blue*.—Dissolve 0.5 gm. of methylene blue in distilled water and make up to 1 liter.

2. Procedure.—If the waste contains caustic alkalinity or acidity, neutralize to bromthymol blue and seed with sewage bacteria. Fill a 150-cc. glass-stoppered bottle with sample, avoiding aëration. Add exactly 0.4 cc. of methylene blue indicator solution below the surface of the liquid. Incubate at 20°C . with a water seal, observing the samples daily until decolori-

zation takes place. Report the days required for decolorization, or if preferred, the relative stability percentage shown in Table 34 or calculated from the formula $S = 100 (1 - 0.794^t)$. If bottles of another size are used add a proportionate amount of indicator. A relative stability of 75 per cent, for example, indicates that the oxygen available is 75 per cent of that required.

TABLE 34.—RELATIVE STABILITY NUMBERS

Time required for decolorization at 20° C., days	Relative stability b. per cent	Time required for decolorization at 20° C., days	Relative stability b. per cent
0.5	11	8.0	54
1.0	21	9.0	57
1.5	30	10.0	60
2.0	37	11.0	62
2.5	44	12.0	64
3.0	50	13.0	65
4.0	60	14.0	66
5.0	68	16.0	67
6.0	75	18.0	68
7.0	80	20.0	69

XI. Biochemical Oxygen Demand.—The biochemical oxygen demand of sewage, sewage effluents, polluted waters and industrial wastes, is the oxygen in parts per million required during stabilization of the decomposable organic matter by aerobic bacterial action. Complete stabilization requires more than one hundred days at 20° C., but such long periods of incubation are impracticable in any but research investigations. Incubation for one, two, five, ten or twenty days at 20° C., is customary, and the five-day period is recommended as the standard procedure. It is essential that normal growth of bacteria and plankton be established in the diluted samples, consequently reliable results cannot be obtained if the diluted sample contains caustic alkalinity, acid, free chlorine or other bactericidal substance. Such substances must be neutralized or removed, and the sample seeded with normal sewage organisms. Filtered sewage should not be used for seeding, since filtration removes protozoa and other organisms necessary for a satisfactory flora.

The dilution water used should have an oxygen demand of less than 0.2 parts per million in five days at 20° C., should contain less than 0.01 p.p.m. of copper, and should be free from residual chlorine, caustic alkalinity, and other bactericidal substances.

1. Reagents and Apparatus.—(a) *Standard Dilution Water.*—Prepare four stock solutions by dissolving the following quantities of reagents in 1-liter quantities of distilled water.

(1) Ferric chloride, 0.25 gm. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$.

(2) Calcium chloride, 11.0 gm. CaCl_2 .

(3) Magnesium sulfate, 10.0 gm. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

(4) Phosphate buffer. Dissolve 34 gm. of potassium acid phosphate (KH_2PO_4) in 200 cc. of distilled water. Add about 175 cc. of N/1 sodium hydroxide until a pH of 7.2 is reached. Then add 1.5 gm. of ammonium sulfate and dilute to 1 liter.

The standard dilution water is prepared by adding to one liter of aerated distilled water, 0.5 cc. of (1), 2.5 cc. of (2), 2.5 cc. of (3), and 1.0 cc. of (4).

(c) *Indicator Buffer.* Dissolve 34 gm. of 20% citric acid, with 2 gm. of glucose, in 1 liter of distilled water. Add 1.0 cc. of 1% sodium carbonate, and dilute to 1 liter.

may be broken by standing, heating, or the addition of solvent to the emulsion.

XV. Chloride.—If the sample contains sulfides, acidify 50 cc. of sample with sulfuric acid, and oxidize the sulfides by heating with hydrogen peroxide for a few minutes. Cool, neutralize with sodium bicarbonate, dilute to the original volume, and proceed as in Chemical Examination of Water, Section XIII.

XVI. Iron.—Proceed as directed in Mineral Analysis of Water, Section II.

XVII. Residual Chlorine.—If nitrite, ferric iron, manganese, or other interfering substances are absent, and equipment is available which permits compensation to be made for the turbidity of the sample, the ortho-tolidine method may be used. The ortho-tolidine solution should contain 180 cc. of concentrated hydrochloric acid rather than 100 cc. For accuracy, the neutral starch iodide method is recommended.

1. **Reagents.**—(a) *Potassium Iodide Solution.*—Dissolve 75 gm. of potassium iodide, free from iodine and iodates, in a small amount of freshly boiled and cooled distilled water. Add 100 cc. ethyl alcohol and make up to 1 liter with freshly boiled and cooled distilled water.

(b) *Sodium Thiosulfate* ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$).—0.01 N Solution. Dissolve 2.483 gm. of C.P. sodium thiosulfate in 1 liter of freshly boiled and cooled distilled water. Add 0.5 cc. of 1 N sodium chromate. One cc. is equiv. to 0.01 N. Add 0.5 cc. of 1 N sodium chromate, if desired. 0.01 N sodium thiosulfate may also be made by dilution from the 0.025 N solution used for dissolved oxygen.

(c) *Starch Solution.*—To 5 gm. of potato starch in a mortar add a little water, mix to a paste, add to 1 liter of boiling distilled water, boil for 5 minutes, and preserve the clear supernatant, and preserve.

2. **Procedure.**—Cool the sample to at least 20° C., preferably lower, and place 1000 cc. in a white porcelain casserole. Add 10 cc. of the potassium iodide solution, stir gently, and allow to stand five minutes. Add 5 cc. of starch solution, and titrate with sodium thiosulfate until the blue color is discharged.

3. **Calculation.**—

$$\text{Residual chlorine, p.p.m.} = \text{cc. of thiosulfate} \times 0.3546$$

XVIII. Chlorine Demand.—1. **Reagents.**—(a) *Chlorine Water.*—By passing chlorine gas through distilled water make a solution containing slightly more than 1 gm. of chlorine per liter. This solution must be standardized every time it is used, preferably by the starch-iodide method.

(b) *Ortho-tolidine Solution.*—As above.

2. **Procedure.**—Measure five or more 250-cc. portions of sample. To one, add chlorine water, 0.5 cc. at a time, with stirring, until a spot plate test using 3 drops of ortho-tolidine to 1 cc. of the sample gives a readable yellow color.

To the other portions, add this quantity of chlorine water, plus increasing amounts in steps of 0.2 to 0.5 cc. Gently stir all flasks and allow to stand for fifteen minutes. Determine the residual chlorine in each flask, using

ortho-tolidine and a colorimeter which permits compensation for turbidity. Calculate from this the chlorine demand, which is the parts per million of chlorine required to produce a residual of 0.1 part per million after fifteen minutes contact.

A similar test may be worked out using the starch-iodide method for determining the residuals.

XIX. pH Value.—For highly colored or turbid sewages and wastes, determination of pH value by electrometric methods is to be preferred. Colorimetric methods may be used for sewages and wastes of moderate color or turbidity or where great accuracy is not desired. Procedures are the same as those for water. Colorimeters used must compensate for the turbidity and color of the sample.

CHAPTER XVIII

MILK, DAIRY PRODUCTS, MEAT AND MEAT PRODUCTS

By **RAYMOND RANDALL** and **JOSEPH N. STABILE**

MILK

I. Physical and Chemical Characteristics.—Milk is the secretion of the mammary glands of female mammals, and is of complex and variable composition. It is a yellowish, opaque fluid, denser than water, containing fat, protein, sugar, salts and smaller amounts of lecithin, urea, and carbon dioxide, all in solution or suspension in water. The milk secreted immediately after parturition differs materially in composition from normal milk; it is known as colostrum. Table 35 is of interest:

TABLE 35.—COMPARATIVE COMPOSITION OF HUMAN AND COWS' MILK (AFTER HOLT)

	Human milk, average per cent	Cows' milk, average per cent
Fat	4 00	3 50
Sugar	7 00	4 30
Protein	1 50	4 00
Ash	0 20	0 70
Water	87 30	87 50

According to Van Slyke and Bosworth, the solid constituents of cows' milk are probably combined as follows:

TABLE 36.—THE SOLIDS OF COWS' MILK

	Per cent
Fat	3 900
Lactose	4.900
Proteins combined with calcium	3 200
Dicalcium phosphate	0 175
Calcium chloride	0 119
Monomagnesium phosphate	0 103
Sodium citrate	0 222
Potassium citrate	0 052
Dipotassium phosphate	0 230
	12 901

The composition of the ash of milk is given as:

TABLE 37.—THE ASH OF MILK

	Per cent
Potassium oxide	25 02
Calcium oxide	20 01
Sodium oxide	10 01
Magnesium oxide	2 42
Iron oxide	0 13
Sulfur trioxide	3 84
Phosphorus pentoxide	24 29
Chlorine	14 28
	100 00

II. Collection and Preparation of Sample—Official.—The quantity of sample required depends upon the number of determinations to be made.

For the usual analysis, 250 to 500 cc. are needed; for fat determinations only, 50 to 60 cc. are sufficient.

In the case of bottled milk, one or more bottles as prepared for sale should be collected. If the milk is in bulk, the sample should be secured only after thorough mixing. This mixing may be accomplished by pouring from one clean vessel into another three or four times or by stirring the milk for at least thirty seconds, with a suitable appliance long enough to reach to the bottom of the container. If cream has formed on the milk continue the mixing until all cream is detached from the sides of the vessel, and evenly emulsified throughout the liquid.

Place the samples in non-absorbent, air-tight containers and keep in the cold, but at a temperature above freezing, until ready for examination. When the sample is to be transported by mail, express, or otherwise, completely fill the containers, tightly stopper, and mark for proper identification. A suitable quantity of preservative such as formaldehyde, 2 drops for each 10 cc. of milk, may be used unless the presence of preservative is objectionable in connection with the physical and chemical tests to be applied in addition to the determination of fat.

Before withdrawing portions for analytical determinations, bring the sample to a temperature of 15° to 20° C. and mix thoroughly by pouring into a clean receptacle and back, until a homogenous mixture is assured. If lumps of cream do not completely disappear, warm the sample to about 38° C., mix thoroughly, then cool to 15° to 20° C. In case a measured volume is required in a determination, bring the temperature of the sample to 20° C. before pipeting.

III. Specific Gravity.—The specific gravity of milk ranges between 1.027 and 1.035. It may be determined by means of the hydrometer. The Quevenne lactometer, with the hydrometer designed includes this range, and m.

close to this temperature are made by specific gravity for each degree above and specific gravity for each degree below 15.6 made within a range of 13° to 18° C.

IV. Acidity.—1. **Reagents.**—(a) *Sodium Hydroxide:* 0.1 N solution. (b) *Phenolphthalein Indicator:* 1 per cent in 50 per cent ethyl alcohol.

2. **Procedure.**—Dilute 10 to 20 cc. of milk with an equal volume of recently boiled and cooled distilled water and titrate with standard sodium hydroxide solution, using phenolphthalein indicator. Express the result as percentage of lactic acid. The determination may be conveniently made by measuring 17.6 cc. of the prepared sample with a 17.6-cc. Balauck pipet, diluting with an equal volume of recently boiled and cooled water, washing out the pipet with carbon dioxide-free water, and titrating with 0.1 N sodium hydroxide solution, using 0.5 cc. of the phenolphthalein indicator.

3. **Calculation.**—The number of cc. of 0.1 N NaOH solution required divided by 20 gives the percentage of lactic acid.

of total solids. The percentage of total solids may also be taken directly from Table 38, interpolations being made where necessary.

If the specific gravity as expressed in Quevenne degrees is a whole number and a decimal, the percentage of total solids corresponding to the whole number is found, and to this is added the fraction found opposite the tenth in Table 39—"Proportional Parts." Two examples are given as illustrations: Fat, 3.8 per cent; specific gravity, 32. Under column headed 32, 12.57 per cent is found corresponding to 3.8 per cent fat. Fat, 3.8 per cent; specific gravity, 32.5. The total solids reading corresponding to this percentage of fat and a specific gravity of 32 is 12.57. Under the Table of Proportional Parts, the fraction 0.13 appears opposite 0.5. This added to 12.57 makes 12.70, which is the desired percentage for the whole number and the decimal.

TABLE 39.—PROPORTIONAL PARTS

Lactometer fraction	Fraction to be added to total solids
0 1	0 03
0 2	0 05
0 3	0 08
0 4	0 10
0 5	0 13
0 6	0 15
0 7	0 18
0 8	0 20
0 9	0 23

An inspection of the table shows that the percentage of total solids increases practically at the rate of 0.25 for each lactometer degree and 1.2 for each per cent of fat. This gives rise to Babcock's simple formula: Total solids equals $\frac{1}{4}$ L plus 1.2 F, where L equals the lactometer reading of fat. To illustrate the Fat, 4 per cent; specific 2 multiplied by 4 is 4.8, 8 plus 4.8 equals 12.8, which represents the percentage of total solids.

VI. Ash—Official.—Into a tared dish pipet about 20 cc. of the prepared sample, weigh quickly, add 6 cc. of nitric acid, evaporate to dryness and ignite at a temperature below redness until the ash is free from carbon. Cool in a desiccator, and report the increase in weight as ash.

VII. Total Nitrogen—Official.—1. Reagents.—(a) *Mercuric Oxide*, or *Mercury, Metallic*.

(b) *Potassium Sulfate*, powdered; or *Sodium Sulfate*, anhydrous.

(c) *Potassium Sulfide*.—Dissolve 40 gm. of commercial potassium sulfide in 1 liter of water.

(d) *Sodium Hydroxide*.—Dissolve 225 gm. of commercial NaOH in 500 cc. of water.

(e) *Hydrochloric or Sulfuric Acid*: 0.1 N.

(f) *Methyl Red Indicator*.

(g) *Sulfuric Acid*: Specific gravity 1.84.

(h) *Sodium or Potassium Hydroxide*: 0.1 N.

2. Procedure.—Transfer 5 gm. of the sample to a Kjeldahl digestion flask. Add approximately 0.7 gm. of mercuric oxide, or its equivalent in metallic mercury, 10 gm. of powdered potassium sulfate, or anhydrous sodium sulfate and 25 to 30 cc. of concentrated sulfuric acid. Place the flask in an inclined position and heat below the boiling-point of the acid

until frothing has ceased. Increase the heat until the acid boils briskly, and digest for a time after the mixture is colorless or nearly so, or until oxidation is complete. Digestion usually requires at least two hours.

After cooling, dilute with about 200 cc. of water, add a few pieces of granulated zinc, pumice stone, or purified talc to prevent bumping and, with shaking, 25 cc. of a solution of potassium sulfide. Next add sufficient sodium hydroxide solution to make the reaction strongly alkaline, 50 cc. are usually sufficient, pouring it down the side of the flask so that it does not mix at once with the acid solution. Connect the flask to the condenser by means of a Kjeldahl connecting bulb, taking care that the tip of the condenser extends below the surface of the standard acid in the receiver; mix the contents by shaking and distil until all ammonia has passed over into a measured quantity of standardized 0.1 N hydrochloric or sulfuric acid. Titrate with standardized 0.1 N alkali solution, using methyl red as an indicator.

3. **Calculation.**—The number of cc. of 0.1 N HCl or H_2SO_4 originally taken minus the number of cc. of 0.1 N alkali used in the titration equals the number of cc. of 0.1 N acid neutralized of the sample. Then this figure multiplied by 6.38 gives the percentage of protein in the milk.

Caution.—Previous to use, the reagents should be tested by a blank experiment with sugar. The sugar partially reduces any nitrates present that might otherwise escape notice.

VIII. Casein—Official.—This determination should be made while milk is fresh, or nearly so. When it is not practicable to make this determination within twenty-four hours, add 1 part of formaldehyde to 2500 parts of milk and keep in a cool place.

1. **Reagents.**—(a) *Acetic Acid.* Dilute.—One cc. of acetic acid plus 9 cc. of water.

2. **Procedure.**—Place 10 gm. of the sample in a beaker with 90 cc. of water at 40° to 42° C. and add at once 1.5 cc. of dilute acetic acid. Stir, and let stand for three to five minutes. Decant on a filter, wash by decantation two or three times with cold water, and transfer the precipitate to the filter. Wash one or two times on the filter. The filtrate should be clear. If the first portions of the filtrate are not clear, repeat the filtration, and complete the washing of the precipitate. Determine the nitrogen in the washed precipitate and filter paper as directed on page 291 and multiply by 6.38 to obtain the equivalent of casein.

To a sample of milk that has been preserved, the acetic acid should be added in small portions, a few drops at a time with stirring, and the addition should be continued until the liquid above the precipitate becomes clear or nearly so.

IX. Albumin—Official.—1. **Reagents.**—(a) *Sodium Hydroxide:* 10 per cent solution.

(b) *Acetic Acid:* Dilute.

2. **Procedure.**—Exactly neutralize the filtrate obtained under VIII with 10 per cent NaOH solution, add 0.3 cc. of dilute acetic acid and heat at once on a steam bath until the albumin is completely precipitated. Collect the precipitate on a filter, wash with cold water, determine the nitrogen

as directed on page 291 and multiply by 6.38 to obtain the equivalent of albumin.

X. Lactose.—Lactose is determined on the protein-free filtrate of milk by a copper reduction method, following the method of Folin-Wu for blood sugar. The method is well suited for routine analyses.

1. **Reagents.**—(a) *Sodium Tungstate*: 10 per cent solution.

(b) *Sulfuric Acid*: 0.66 N.

(c) *Standard Lactose Solution*.—Prepare a stock standard by dissolving 1 gm. of lactose in 0.25 per cent benzoic acid solution and making up to a volume of 100 cc. The working standard is made up by diluting 3 cc. of this stock solution to 100 cc. with 0.25 per cent benzoic acid. In this working standard 2 cc. equals 0.6 mg. of lactose.

(d) *Alkaline Copper Reagent*.—Same as Chapter XVI, page 207.

(e) *Molybdate-phosphate Solution*.—Same as Chapter XVI, page 208.

2. **Procedure.**—Introduce 1 cc. of milk into a 100-cc. volumetric flask, add 2 cc. of 10 per cent sodium tungstate and mix well. Add gradually 2 cc. of 0.66 N H_2SO_4 , mix well and let stand five minutes. Dilute to volume with water and filter. Introduce 1 cc. of the filtrate and 1 cc. of water into a Folin sugar tube. In another tube, place 2 cc. of the standard lactose solution.

Add 2 cc. of the alkaline copper reagent to each tube and heat in a boiling water bath for eight minutes. Cool and add 4 cc. of acid molybdate solution to each tube. After one minute, add diluted molybdate solution, 1 + 4, to the 25-cc. mark. Mix and compare in the colorimeter.

3. **Calculation.**—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.6 \times \frac{100}{0.01} \times \frac{1}{1000} = \text{per cent}$$

lactose or, with the unknown set at 20 mm., Reading of the Standard $\times 0.3$ = per cent Lactose.

XI. Fat. A. Roese-Gottlieb Method—Official.—1. **Reagents.**—(a) *Ammonium Hydroxide*.

(b) *Ethyl Alcohol*: 95 per cent.

(c) *Petroleum Ether*.—Redistilled slowly at a temperature below 65° C.

(d) *Ethyl Ether*, anhydrous.

2. **Procedure.**—Transfer 10 gm. of the sample to a Rohrig tube or similar apparatus, add 1.25 cc. of ammonia water, or 2 cc. if the sample is sour, and mix thoroughly. Add 10 cc. of 95 per cent alcohol and mix. Add 25 cc. of ethyl ether, shake vigorously for thirty seconds, add 25 cc. of petroleum ether and again shake thirty seconds. Let stand twenty minutes. Draw off as much as possible of the ether-fat solution into a flask through a small, quick-acting filter. Again extract the liquid remaining in the tube, this time with 15 cc. of each ether. Draw off the clear solution as before. To insure complete removal of the fat a third extraction is necessary. This third

weight. Weigh the flask with a similar flask as a counterpoise. Do not wipe the flask immediately before weighing. Remove the fat completely with petroleum ether. Deduct the weight of the dried flask with residue and bead to obtain the weight of fat. Finally, correct this weight by a blank determination on the reagents used.

B. Babcock Method—Official.—1. Apparatus and Reagents.—(a) *Test Bottles (Babcock).*—These test bottles must conform to the specifications as given in "Standard Methods for the Examination of Dairy Products," American Public Health Association, 8th Edition, 1941.

(b) *Centrifuge.*—Speed 600 to 1200 r.p.m.

(c) *Pipet.*—17.6 cc. and 17.5 cc. graduations only, conforming to specifications.

(d) *Sulfuric Acid:* Specific gravity 1.82 to 1.83 at 20° C.

2. Procedure.—Transfer 17.6 cc. of the milk sample to the test bottle by means of the pipet. Blow out the milk remaining in the pipet tip after free outflow has ceased. Add 17.5 cc. of sulfuric acid, preferably not all at one time, pouring it down the side of the neck of the bottle in such a way as to wash any traces of milk into the bulb. The temperature of the acid should be about 15° to 20° C. Shake until all traces of curd disappear; then transfer the bottle to a centrifuge, counterbalance it, and whirl for five minutes after the proper speed has been attained. Add soft or distilled water at 60° C. or above until the bulb of the bottle is filled. Whirl two minutes. Add hot water until the liquid approaches the top graduation on the scale. Whirl one minute. Transfer the bottle to a water bath at 55° to 60° C., immerse it to the level of the top of the fat column and leave it there until the column is in equilibrium and the lower fat surface has assumed a final form. Remove the bottle and, with the aid of calipers or dividers, measure the column of fat in terms of percentage by weight, from its lowest surface to the highest point of the upper meniscus.

The fat column, at the time of measurement, should be translucent, of a golden yellow or amber color, and free from visible suspended particles. Reject all tests in which the fat column is milky or shows the presence of curd or charred matter, or in which the reading is indistinct or uncertain.

XII. Added Water (Acetic Serum—Ash Method)—Official.—1. Reagents.—(a) *Acetic Acid.*—Twenty-five per cent solution (specific gravity 1.035).

2. Procedure.—To 100 cc. of the milk measured at 20° C. into a beaker, add 2 cc. of 25 per cent acetic acid. Cover the beaker with a watch glass and place it in a water bath at 70° C. for twenty minutes. Cool the beaker in ice water for ten minutes and separate the curd from the serum by rapid filtration through a small filter.

Transfer 25 cc. of the serum to a tared, flat-bottomed platinum dish and evaporate to dryness on a water bath. Heat over a low flame until the contents are thoroughly charred, place the dish in an electric muffle, preferably with pyrometer attached, and ignite to a white ash at a temperature not greater than 500° C. Cool and weigh.

3. Calculation.—Express the result as gm. per 100 cc. A result below 0.715 gm. per 100 cc. indicates added water. The serum ash, multiplied by the factor 1.021 equals the sour serum ash, dilution of the acetic serum being 2 per cent.

XIII. Gelatin. Qualitative Test—Official.—1. Reagents.—(a) *Acid Mercuric Nitrate.*—Mercury is dissolved in twice its weight of nitric acid, and this solution diluted to twenty-five times its volume with water.

(b) *Picric Acid.*—Saturated solution.

2. Procedure.—To 10 cc. of the milk add an equal volume of acid mercuric nitrate solution, shake the mixture, add 20 cc. of water, shake again,

allow to stand five minutes, and filter. If much gelatin is present, the filtrate will be opalescent and cannot be obtained quite clear. To a portion of the filtrate in a test tube add an equal volume of saturated picric acid solution. A yellow precipitate will be produced in the presence of any considerable quantities of gelatin, while smaller quantities will be indicated by a cloudiness. In the absence of gelatin, the filtrate will remain perfectly clear.

XIV. Preservatives. A. Formaldehyde.—1. **Leach Test**—Official.—Mix about 10 cc. of milk with an equal volume of strong hydrochloric acid containing 1 cc. of 10 per cent ferric chloride solution to each 500 cc. of acid. Heat slowly in a casserole to 80° to 90° C. directly over a gas flame, rotating the casserole to break up the curd. A violet color indicates formaldehyde.

2. **Phenylhydrazine Hydrochloride and Sodium Nitroprusside Test**—Official.—Dissolve a lump of phenylhydrazine hydrochloride the size of a pea in 3 to 5 cc. of the milk to be tested, add 2 to 4 drops, not more, of a 5 to 10 per cent solution of sodium nitroprusside and 8 to 12 drops of an approximately 10 per cent sodium hydroxide solution. A green or blue color indicates formaldehyde. When present to the extent of more than 1 to 70,000 in the solution tested, a distinct green or bluish-green coloration results. In more dilute solutions the green tint is less marked and a yellow tinge tending toward greenish-brown develops.

3. **Phenylhydrazine Hydrochloride and Potassium Ferricyanide Test**—Official.—Proceed as above, substituting a solution of potassium ferricyanide for the sodium nitroprusside. Formaldehyde gives a red color.

4. **Hehner Test**—Official.—To about 10 cc. of the milk in a wide test tube, add about one-half its volume of commercial sulfuric acid, pouring the acid carefully down the side of the tube so that it forms a layer at the bottom without mixing with the milk. A violet or blue color at the junction of the two liquids indicates formaldehyde. The test is given only in the presence of ferric chloride or other oxidizing agents. This test may be combined with the Babcock test for fat, noting whether a violet color forms upon the addition of the commercial H_2SO_4 to the milk in the test bottle.

B. Salicylic Acid.—1. **Ferric Chloride Test**—Official.—Acidify 100 cc. of the milk with 5 cc. of hydrochloric acid, 1 to 4. Shake until curdled, filter and extract the filtrate with 50 to 100 cc. of ether. Wash the ether layer with two 5-cc. portions of water, evaporate the greater portion of the ether in a porcelain dish on the water bath, allow the remainder to evaporate spontaneously and add a drop of 0.5 per cent ferric chloride solution. A violet color indicates salicylic acid.

2. **Jorissen Test**—Official.—Acidify, filter and extract a portion of the milk as above, or divide the ether extract above into two portions, using one for the ferric chloride test, the other for this test. Evaporate the ether extract to dryness. Dissolve the residue in a little hot water. Cool 10 cc. of this solution in a test tube, add 4 or 5 drops of a 10 per cent potassium nitrite solution, 1 or 5 drops of 50 per cent acetic acid and 1 drop of a 1 per cent cupric sulfate solution, mix thoroughly and heat to boiling. Boil for thirty seconds and let stand one to two minutes. In the presence of salicylic acid, a blood-red color will develop.

C. Benzoic Acid—Official.—Prepare an ether extract as above. If benzoic acid is present in considerable quantity it will crystallize from the ether in shining leaflets giving a characteristic odor on heating.

Dissolve the ether residue in hot water, divide it into two portions and apply the following tests:

1. Make the solution alkaline with ammonium hydroxide, expel the excess of ammonia by evaporation, dissolve the residue in water and add a few drops of a neutral 0.5 per cent ferric chloride solution. A brownish precipitate of ferric benzoate indicates the presence of benzoic acid.

2. Add to the second aqueous portion 1 or 2 drops of a 10 per cent solution of sodium hydroxide and evaporate to dryness. To the residue add 5 to 10 drops of concentrated sulfuric acid and a small crystal of potassium nitrate. Heat for ten minutes in a glycerol or oil bath at 120° to 130° C. or for twenty minutes in a boiling water bath. The temperature must not exceed 130° C. Cool, add 1 cc. of water and make distinctly ammoniacal; boil to decompose any ammonium nitrate formed. Cool and add fresh colorless ammonium sulfide without allowing the layers to mix. A red-brown ring indicates benzoic acid. On mixing, the color diffuses throughout the liquid and on heating changes to greenish-yellow.

D. Boric Acid.—1. Preliminary Test—Official.—Immerse a strip of turmeric paper in the sample acidified with hydrochloric acid in the proportion of 7 cc. of strong acid to each 100 cc. of sample, and allow the paper to dry spontaneously. If borax or boric acid is present, the paper will acquire a characteristic red color, changed by ammonium hydroxide to a dark blue-green, but restored by acid. If this preliminary test is positive, proceed with the

2. Confirmatory Test—Official.—Make about 25 cc. of the sample decidedly alkaline with lime water and evaporate to dryness on a water bath. Ignite the residue at a low red heat to destroy organic matter. Digest with about 15 cc. of water, add strong hydrochloric acid, drop by drop, until the ignited residue is dissolved, and then add 1 cc. in excess. Saturate a piece of turmeric paper with the solution and allow it to dry without the aid of heat. In the presence of borax or boric acid, the color change will be the same as in the preliminary test.

XV. Coloring Matters.—1. Annatto—Official.—Warm about 150 cc. of milk in a casserole over a flame and add about 5 cc. of 25 per cent acetic acid, then slowly continue the heating nearly to the boiling point while stirring. Gather the curd, when possible, into one mass with a stirring rod, and pour off the whey. If the curd breaks up into small flecks, separate from the whey by straining through a sieve. Press the curd free from the adhering liquid, transfer to a small flask, macerate, allow to stand for several hours in about 50 cc. of ether, keeping the flask tightly corked and shaking at intervals. Decant the ether extract, evaporate the ether on the water bath, make the residue alkaline with NaOH, and pour upon a small wet filter. If annatto is present, the filter paper will absorb the color so that, when washed with a gentle stream of water, it will remain dyed a straw color. Dry the filter and add a drop of stannous chloride solution. If the color turns pink, the presence of annatto is confirmed.

2. Coal Tar Dyes—Official.—The curd of an uncolored milk is perfectly white after complete extraction with ether, as is also that of milk with annatto. If the extracted fat-free curd is distinctly orange or yellowish in color, a coal tar dye is indicated. In many cases upon treating a lump of a fat-free curd in a test tube with a little hydrochloric acid, the color

changes to pink, indicating the presence of a dye similar to aniline yellow or butter yellow or perhaps one of the acid azo yellows or oranges.

In some cases the presence of coal tar dyes can be detected by heating 100 cc. of milk directly with an equal volume of HCl in a porcelain casserole, giving the dish a slight rotary motion. In the presence of some dyes, the separated curd acquires a pink coloration.

XVI. Raw and Heated Milk.—The Rapid Phosphatase Test.—The phosphatase test is based on the property of the heat-sensitive enzyme phosphatase to liberate phenol from phosphoric-phenyl esters. When milk is heated, this enzyme becomes progressively inactivated, 96 per cent of it being destroyed when heated at 143° F. for thirty minutes; when heated above 145° F. for the same length of time it is completely inactivated.

1. **Reagents.**—(a) *Neutral n-butyl alcohol*, boiling range 115° to 118° C.

(b) *Gibbs' Phenol Reagent*.—Dissolve 40 mg. of 2, 6-dibromoquinonechlorimide in 10 cc. of methyl or 95 per cent ethyl alcohol. Keep the reagent tightly stoppered and under refrigeration.

(c) *Borate Buffer*.—Dissolve 28.427 gm. of sodium borate, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, in 900 cc. of water. Add 3.27 gm. of sodium hydroxide or 81.75 cc. of 1 N NaOH and dilute to 1 liter.

(d) *Buffer Substrate*.—Dissolve 0.5 gm. of crystalline disodium phenyl phosphate in 5 cc. of water in a small test tube. Add 0.5 cc. of borate buffer. Shake well and add 0.05 cc. of the Gibbs' phenol reagent. Shake well and allow five minutes for color development. Extract indophenol by shaking with 2 cc. of neutral n-butyl alcohol and allow to stand until the alcohol has separated completely. Remove the supernatant alcohol layer with a pipet and discard. Dilute the remainder with 100 cc. of borate buffer and add water to make 1 liter. Store under refrigeration and prepare only enough for immediate use. Avoid contact of the solution with rubber.

(e) *Color Standards*:

(1) Color solution, red—0.5 N cobalt chloride in 1 per cent HCl (59.59 gm. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ per liter).

(2) Color solution, blue—0.5 N copper sulfate in 1 per cent HCl (62.425 gm. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per liter).

(3) Color solution, yellow—0.5 N (M/6) ferric chloride in 1 per cent HCl (45.05 gm. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ per liter).

Combine the quantities indicated below and dilute to 5 cc.

Units	Red, cc.	Blue, cc.	Yellow, cc.
2	0 4	1 5	0 5
5	0 2	2 2	0 5

2. **Procedure.**—To a test tube (10 x 110 mm.) calibrated at 5 cc., 5.5 cc., and 7.5 cc. measured to top of meniscus and fitted with gum rubber stoppers, add 5 cc. of buffered substrate and 0.5 cc. of milk sample. Shake briefly. Incubate for ten minutes in a water bath at 36° to 44° C. If no water bath is available, incubate in pocket for a fifteen to twenty minute period. After incubation add 6 drops of Gibbs' phenol reagent and shake well immediately. Allow to stand for five minutes. Appearance of a blue color indicates inadequate pasteurization. For greater sensitivity add 2 cc. of neutral n-butyl alcohol. Extract the indophenol by completely inverting the tube

ten times, pausing after inversion to allow bubbles to break and alcohol to separate. Compare with color standards.

3. **Interpretation of Results.**—A value equal to or greater than 2 units generally indicates faulty pasteurization. The test tube containing the standards must be of the same size as that containing the sample being tested.

XVII. Fat in Human Milk.—For this determination the Babcock method is employed, making use of a special small test bottle of the same type as the Babcock bottle; the body holds about 8 cc. and bears a mark at the 5-cc. point, while the narrow neck is marked from 0 to 5 per cent in 0.2 per cent divisions.

Add milk to the 5-cc. mark, making use of a narrow-tipped pipet. Then fill the body of the bottle by adding about 3 cc. of concentrated sulfuric acid. Mix and add a few drops of a mixture of HCl and amyl alcohol, to bring the total volume to the zero mark. Centrifugalize for two minutes and read the layer of fat, now up in the stem.

This method gives results that check within 0.5 per cent with the more accurate methods and is quite satisfactory for clinical purposes.

Milks of higher fat content than 5 per cent must be diluted with an equal volume of water, and the result multiplied by 2.

XVIII. Lactose in Human Milk.—Proceed as directed on page 293.

CREAM

I. Collection and Preparation of Sample—Official.—Proceed as under Milk, page 288. The sample should be analyzed as soon as practicable, and preferably not later than three days after taking.

Immediately before withdrawing portions for the determinations, mix the sample by shaking, pouring or stirring until it pours readily and a uniform emulsion has been secured. If the sample is very thick, warm it to 30° to 35° C. and then mix. In cases where lumps of butter have separated, heat the sample to 38° C. or, if necessary, to 50° C. by placing it in a warm water bath. Thoroughly mix the portions for analysis and weigh immediately. Avoid overheating the sample, thereby causing the cream to "oil off." This precaution is especially necessary in the case of a thin cream.

II. Total Solids—Official.—Proceed as directed under Milk, page 290, using 2 to 3 gm. of the sample.

III. Ash—Official.—Proceed as directed under Milk, page 291.

IV. Total Nitrogen—Official.—Proceed as directed under Milk, page 291.

V. Lactose.—Proceed as directed under Milk, page 293.

VI. Fat.—1. **Röse-Gottlieb Method—Official.**—Transfer 5 gm. of the
with water to about

that employed for milk. Either a 9-gm. or 18-gm. test bottle may be used.

Weigh 9 or 18 gm. of the sample directly into a 9-gm. or 18-gm. cream test bottle and add 8 to 12 cc. of sulfuric acid in the case of a 9-gm. bottle or 14 to 17 cc. of this acid in the case of an 18-gm. bottle and proceed as outlined under Milk, page 294.

VII. Acidity.—1. **Reagents.**—(a) *Sodium Hydroxide*: 0.1 N NaOH solution.

(b) *Phenolphthalein*: 1 per cent phenolphthalein solution in 50 per cent ethyl alcohol.

2. *Procedure*.—Weigh out exactly 9 gm. of cream and dilute with 27 cc of CO₂ free water. Add 0.5 cc. of phenolphthalein indicator and titrate with 0.1 N NaOH until a faint pink color remains.

3. *Calculation*.—The number of cc. of 0.1 N NaOH divided by 10 will give the per cent of acidity calculated in terms of lactic acid.

VIII. *Gelatin*—Official.—Proceed as directed under Milk, page 294.

IX. *Preservatives*—Official.—Proceed as directed under Milk, page 295.

X. *Coloring Matters*—Official.—Proceed as directed under Milk, page 296.

ICE CREAM

I *Preparation of Sample*—Official.—Allow the sample to soften at room temperature. Owing to the fact that melted butter fat tends to separate out and rise to the surface, it is not advisable to soften the ice cream by heating on a water bath or over a flame. Mix thoroughly by stirring with a spoon or an egg beater or by pouring back and forth between beakers.

II. *Total Nitrogen*.—Place 4 to 5 gm. of the sample in a digestion flask and proceed as directed under Milk, page 291.

III. *Fat. Roesse-Gottlieb Method*—Official.—Weigh 4 gm. of the thoroughly mixed sample into a small, dry beaker, add 3 cc. of water; thoroughly mix by stirring with a glass rod, and transfer to a Rohrig tube, washing out the remaining portion with the aid of an additional 3 cc. of water. Add 2 cc. of ammonia, mix thoroughly and heat in a water bath at 60° C. From this point proceed as directed under Milk, page 293, beginning with "Add 10 cc. of 95 per cent alcohol and mix well."

IV. *Coloring Matters*.—Curdle 150 to 200 gm. of the melted sample by adding an equal volume of water and 10 to 20 cc. of acetic acid. Heat the mixture to 70 to 80° C., stirring meanwhile and allow to cool. Continue as directed under Milk, page 296.

BUTTER

I. *Physical and Chemical Characteristics*.—Butter is composed of the various fatty acid glycerides of milk together with water, salt, casein and sugar. The average composition, physical characteristics and related values of butter are shown in Tables 40 and 41.

TABLE 40.—AVERAGE COMPOSITION OF BUTTER

	Per cent
Water	12 00
Fat	85 00
Salt and ash	2 25
Casein and sugar	0 75

TABLE 41.—PHYSICAL CONSTANTS AND RELATED VALUES

Specific gravity	0 865 to 0 870 (100° C)
Melting-point	28 to 36° C.
Butyro refraction	40 5 to 40
Refractive index	1 4527 to 1 4566
Saponification number	220 to 241
Iodine number	26 to 38
Reichert-Meissl	24 to 34

The U. S. Government requires a minimum of 82.5 per cent fat; minimum allowable specific gravity is 0.905 at 40° C., compared with that of water

at the same temperature. The U. S. Government minimum Reichert-Meissl number is 24.

Oleomargarine is a mixture of fats, artificially colored to resemble butter. Oleomargarine has the sanction of the U. S. Government, but "butterine" has not. Its average composition is:

TABLE 42.—AVERAGE COMPOSITION OF OLEOMARGARINE

	Per cent
Oleo oil	20 to 25
Lard, neutral	40 to 45
Butter	10 to 25
Milk, cream, salt, etc.	5 to 30

Oleomargarine often contains cottonseed, coconut, palm kernel and other oils.

Renovated, or processed butter, is the third type recognized by the U. S. Government; this is often referred to by the names of boiled, aerated and sterilized butter.

This is rancid butter that has been heated in hot-water jackets to between 40° and 45° C.; this causes the curds and brine to settle to the bottom and a frothy foam to rise to the top. These are removed and air is blown through the melted mass to remove the disagreeable odors. The melted fat is then churned with milk, usually skimmed, until a perfect emulsion is formed, then chilled. It is next allowed to drain and is "ripened" for several hours, then is worked free from excess milk and water, and finally salted and packed. The U. S. Government requirements for this type of butter are: A minimum of 82.5 per cent butter fat, and a maximum of 16 per cent water.

II. Collection and Preparation of Sample.—By far the greatest discrepancies found in the analyses of various observers with regard to water content are caused by inefficient sampling and after-care of the sample. In sampling large bulks of butter or margarine, a piece 2 inches long by 1 inch square is cut by means of a thin wire from the center of the mass, and the piece so cut lightly pushed with the finger into a dry 200-cc., wide-mouth bottle. The bottle is then corked and the sample is placed in a water bath. A well-ventilated water bath is well adapted for this purpose. The water from the sample when melted may pass between the stopper and the neck by capillary attraction. The bottles containing the samples are placed in water, or preferably in an incubator, at 38° to 40° C. until melted.

III. Water—Official.—Into a flat-bottomed dish, place about 10 gm. of water. Weigh this dish with water accurately about 10 gm. and place in a water bath. Allow to cool and when the weight is obtained. Then

$$\frac{\text{Loss of weight (in gm.)}}{\text{Weight of sample (in gm.)}} \times 100 = \text{percentage of water present}$$

IV. Salt—Official.—Weigh in a counterpoised beaker 5 to 10 gm. of butter or margarine; add about 20 cc. of hot water and after the butter has melted, transfer the whole to a separatory funnel. Shake for a few minutes, allow to stratify, then draw off the water layer, being careful that none of

the fat globules pass. Again add hot water, rinsing the beaker, and repeat the extraction ten to fifteen times, using 10 to 20 cc. of hot water each time. Mix the washings and determine the sodium chloride in the entire quantity or in an aliquot part by titrating with 0.1 N silver nitrate solution, using potassium chromate as an indicator.

V. *Ash*.—Ignite the residue obtained above (III, Water, page 300) until a constant weight is obtained.

$$\frac{\text{Weight in ash}}{\text{Weight of sample}} \times 100 = \text{per cent of ash}$$

VI. *Fat*.—1. *Röse-Gottlieb Method*.—Proceed as outlined under Milk, page 293.

2. *Direct Method—Official*.—Extract the fat from the dry butter obtained in the moisture determination above, page 300, with anhydrous alcohol-free ether, or with petroleum ether, boiling-point below 65° C., receiving the solution in a weighed flask. Evaporate the ether, dry the extract at 100° C. and weigh at hourly intervals until the weight is constant.

VII. *Total Nitrogen (Curd)*.—Proceed as outlined under Milk, page 291.

VIII. *Mold Mycelia*.—Butter differs from most other dairy products in that cultures of lactic acid organisms are frequently added to cream. As a guide to determine the quality of cream from which butter was manufactured the microscopic mold count test may be employed. The presence of the dead mold mycelia in more than 60 per cent of the fields is considered indicative of filthy, putrid or decomposed cream having been used in its manufacture.

1. *Apparatus and Reagents*.—(a) *Compound Microscope*.—Equipped with good objectives of about 90, 180, and 500 diameters. It is essential that the combination be capable of adjustment to give an area of a field of view of 1.5 sq. mm. (a circle whose diameter is 1.382 mm.).

(b) *Drop-in Cross-ruled Disk*.—For estimating lengths of mold filaments, an ocular drop-in disk cross-ruled in sixths of the ocular diaphragm opening is desirable.

(c) *Howard Mold-counting Cell*.—Constructed like the blood-counting cell but with unruled central disk about 19 mm. in diameter.

(d) *Gum Solution*.—Make up 1 liter of 0.75 per cent solution of carob bean gum or 1 per cent gum tragacanth with 2 per cent of added formaldehyde as a preservative. The dry gum may be conveniently dissolved by first mixing it in 10 to 15 cc. of alcohol and stirring this mixture rapidly into the water. Gently heat the solution to boiling to drive off the alcohol and air, and continue heating for twenty-five to thirty minutes. Add the formaldehyde after cooling. Use the clear supernatant solution, free from cells, left when the cellular elements in gum gradually settle out.

(e) *Crystal Violet Solution*.—Five per cent aqueous solution.

2. *Procedure*.—To avoid contamination with any surface mold scrape off and discard $\frac{1}{4}$ inch of surface. Take the sample from the newly exposed surface and weigh 1 gm. of butter in a $\frac{1}{4}$ teaspoon measure. Measure out 7 cc. of the hot gum solution, and with spoon bottom side up over a 50-cc. beaker, pour 2 or 3 cc. of the hot gum solution to loosen butter. Use the remainder of the hot gum solution to rinse the fat from the spoon. Add

1 or 2 drops of crystal violet solution and stir until the mixture is uniform and fat globules are 0.1 to 0.2 mm. in diameter.

Clean the special Howard cell so that Newton's rings are produced between slide and cover glass. Remove cover and place a small drop of the well mixed sample upon the central disk; using a knife blade or scalpel, spread the drop evenly over the disk and cover so as to give an even spread.

It is of utmost importance that the drop be taken from a thoroughly mixed sample and spread evenly over the slide disk. Otherwise, when the cover slip is put in place the insoluble material, and consequently the molds, may be more abundant at the center of the mount. Avoid using a drop that is much greater than is sufficient to fill the space between center disk and cover slip. Discard any mount showing uneven distribution, absence of Newton's rings, or liquid that has been drawn across moat and under cover glass.

Place the slide under the microscope and examine with such adjustment that each field of view covers 1.5 sq. mm. When the instrument is properly adjusted, the quantity of liquid examined per field is 0.15 cmm. (0.00015 cc.).

From each of two or more mounts examine at least 25 fields taken in such a manner as to be representative of all sections of the mount. Observe each field, noting presence or absence of mold filaments and recording

3. Calculation.—Calculate the proportion of positive fields from the results of examination of all observed fields and report as percentage of fields containing mold filaments.

IX. Preservatives.—In the past, boron compounds have been the most commonly used preservatives in butter and margarine.

Melt a portion of the sample, about the size of a pea, in a small crucible lid on the water bath, add 1 drop of strong hydrochloric acid, stir with a glass rod and add 5 to 6 drops of a saturated solution of turmeric. A rose-red color, immediately forming as the edges dry, indicates the presence of boron compounds, the color changing to a purple-green upon the addition of 1 drop of ammonia. This method will detect 0.02 per cent boric acid with certainty.

For other preservatives, proceed as under Milk, page 295.

X. Coloring Matters.—The complete examination for coloring matter in butter involves a complex examination outside the scope of this work and reference should be made to the texts listed at the end of this Chapter.

The following simple methods, however, will give useful information as to the general nature of coloring matters in butter and margarine:

1. Annatto.—Shake a few gm. of clear filtered fat with about 5 cc. of warm 10 per cent sodium hydroxide solution. Filter the mixture, keeping the filter and mixture warm. Pour the melted fat off the filter paper and gently wash with cold water. In the presence of annatto, the paper will be stained a reddish-yellow of an intensity dependent upon the quantity of annatto present. Dry the filter paper and moisten with 1 drop of a 5 per cent solution of citric acid; a pink color will be produced even when there is not sufficient annatto to produce a definite yellow stain.

2. **Azo-Dyes.**—(a) *Doolittle Method.*—Place 2 gm. of the filtered fat in each of 2 test tubes and add 5 cc. of petroleum ether to prevent solidification of the fat. To one tube add 1 cc. of hydrochloric acid, diluted 1 to 3; to the other tube add 1 cc. of 10 per cent potassium hydroxide solution; shake both tubes thoroughly and allow to stand. If an azo-dye be present, the lower layer in the hydrochloric acid tube will be pink, while that in the potassium hydroxide tube will remain colorless.

(b) *Low Method.*—Mix 1 cc. of the melted fat with 1 cc. of a mixture of 1 part of concentrated sulfuric acid and 4 parts of glacial acetic acid and heat to the boiling-point with constant shaking. In the presence of azo-dyes the acid solution on settling out will be colored pink or reddish.

XI. Added Foreign Fats.—In determining whether the fat present in a butter is pure butter fat, or whether foreign fats or oils have been added, several tests are of value. Examination is usually made for the presence of margarine fats. Coconut oil is probably seldom used, *per se*, but on account of the use of large quantities of this fat and of palm kernel oil in many modern margarines, these may be found as adulterants. On account of the wide variations in the figures obtained for pure butter fat, the greater number of tests are of little or no value by themselves and have only a cumulative significance. It is necessary, therefore, to employ those tests which will yield the most valuable information. The indications derived from the determination of the Reichert-Meissl-Polenske-Kirschner figures and of the baryta value by the method of Avé Lallemand, supported by qualitative tests, will give all the information with regard to adulteration of which present analytical methods are capable.

TABLE 43—CONSTANTS OF BUTTER FAT AND OLEOMARGARINE

	Sp. gr at 100° C	Hehner number	Saponi- fication number	Reichert- Meissl number	Refractive index at 35° C
Butter fat maximum	0 870	89 6	233	34 86	1 4578
Butter fat minimum	0 867	85 6	222	22 70	1 4557
Oleomargarine maximum	0 862	95 5	203	5 50	1 4625
Oleomargarine minimum	0 858	92 5	192	0 50	1 4613

TABLE 44—EFFECT OF ADDED FOREIGN FATS

	Hehner number	Saponi- fication number	Reichert- Meissl number
Pure butter	88 00	224 0	26 0
Butter, 95%; foreign fat, 5%	88 35	222 6	24 7
Butter, 90%; foreign fat, 10%	88 70	221 2	22 2
Butter, 85%; foreign fat, 15%	89 05	219 8	20 9
Butter, 80%; foreign fat, 20%	89 40	218 4	19 6
Butter, 75%; foreign fat, 25%	89 75	217 0	18 3

A. Reichert-Meissl-Polenske-Kirschner Values.—This process is based

of glycerides of these water-insoluble volatile acids, shows a small Polenske value relative to the Reichert-Meissl value whereas coconut products, which are largely made up of them, give a high Polenske value. The addition of coconut or palm kernel oil or their products to butter, therefore, depresses the Reichert-Meissl value and decidedly raises the Polenske value.

1. Reagents.—(a) *Glycerol*.

(b) *Sodium Hydroxide*: 50 per cent solution.

(c) *Sulfuric Acid*.—Sixty gm. diluted to 1000 cc. and the solution adjusted so that 35 cc. neutralizes 2 cc. of the concentrated sodium hydroxide.

(d) *Barium Hydroxide*: 0.1 N.

(e) *Ethyl Alcohol*: 95 per cent.

(f) *Silver Sulfate*.

(g) *Phenolphthalein Indicator*: 1 per cent solution.

2. Procedure.—(a) *Reichert-Meissl Value*.—Weigh 5 gm. of the sample and 20 gm. of glycerol into a 300-cc. flask and add 2 cc. of concentrated sodium hydroxide solution. Heat the flask over a flame, with constant shaking, until the contents clear suddenly. Allow the flask to cool somewhat, and add 100 cc. of recently well-boiled, distilled water. Arrange a distillation apparatus with the condenser placed vertically and connect the flask immediately after adding 0.1 gm. of powdered pumice, and 40 cc. of the sulfuric acid solution. Heat over a small flame until the insoluble acids are completely melted; then increase the flame and distil 110 cc. in nineteen to twenty-one minutes. After the 110 cc. have been distilled, remove the flame and place a 25-cc. cylinder to catch any drops from the condenser. Cool the distillate in water at 10° to 15° C. for fifteen minutes. Filter and titrate 100 cc. with 0.1 N barium hydroxide using 0.1 cc. of phenolphthalein indicator.

(b) *Polenske Value*.—Wash the condenser, cylinder and 110-cc. flask with 18 cc. of cold water; pour this over the filter used to filter the distillate and reject. Now wash the condenser with four successive portions of 10 cc. of neutral 95 per cent ethyl alcohol. Pour this over the filter and receive it in the 110-cc. flask. Titrate the mixed alcoholic solutions with 0.1 N

(c) *0.1 N* sulfate. Allow to stand for one hour with occasional shaking. Filter, measure off 100 cc. and add 35 cc. of water and 10 cc. of sulfuric acid solution together with a long piece of aluminum wire. Again distil 110 cc. Titrate 100 cc. of this distillate with 0.1 N barium hydroxide, using phenolphthalein as an indicator.

Blank determinations on all reagents must be run in an identical manner using 5 gm. of water instead of the sample, following in detail the procedures above.

3. Calculation.—The number of cc. of 0.1 N barium hydroxide used in the first titration, increased by one-tenth, after subtraction of the blank determination, is the Reichert-Meissl value.

The number of cc. of 0.1 N barium hydroxide used, less the number used for the blank, in the second titration gives the insoluble fatty acids or the Polenske value.

The number of cc. used, corrected for the blank, in the third titration is calculated to the Kirschner value by the following formula:

$$K = \frac{x \cdot 121 (100 + y)}{10,000}$$

where x = the corrected Kirschner titration and

y = the number of cc. of 0.1 N barium hydroxide solution used to neutralize the 100 cc. of solution used in the first titration.

NOTE.—The use of benzoic acid as a preservative in butter or margarine has a decided effect upon the above values. This effect may be removed by washing the fat with a solution of sodium bicarbonate. Shake violently 50 gm. of the melted sample for five minutes with 150 cc. of a 5 per cent sodium bicarbonate solution. After the fat separation, wash once with 150 cc. of warm water. Filter and render the fat water-free.

B. Saponification Number. Koettstorfer Number.—The saponification number of a fat or of an oil is the number of milligrams of potassium hydroxide consumed in saponifying 1 gm. of that fat or oil.

1. **Reagents.**—(a) *Alcoholic Potassium Hydroxide:* 0.5 N (1 cc. = 0.028055 gm. KOH). Purify the alcohol as follows: Dissolve 2.5 gm. of silver nitrate in 5 cc. of distilled water and add to it 1200 cc. of alcohol. Dissolve 5 gm. of potassium hydroxide in 25 cc. of warm alcohol, cool and add slowly, without agitation, to the silver nitrate solution. Allow to stand overnight, or until the silver oxide precipitate has completely settled. Filter and distil off the alcohol.

Dissolve 35 gm. of potassium hydroxide in 20 cc. of distilled water and add sufficient purified alcohol to make 1 liter. Stopper with a rubber stopper. Allow to stand in a cool, dark place for twenty-four hours. Decant the clear liquid and titrate against 0.5 N hydrochloric acid, using phenolphthalein as an indicator, using as the end point the production of a red color that persists for two minutes. Adjust the volume of this solution according to this titration. This solution must be protected against the possibility of absorbing carbon dioxide.

(b) *Hydrochloric Acid:* 0.5 N.

(c) *Phenolphthalein Indicator:* 1 per cent alcoholic solution.

2. **Procedure.**—In a flask of 200 to 250-cc. capacity weigh accurately 1.5 to 2 gm. of fat or oil under examination, add 25 cc. of the alcoholic potassium hydroxide solution and boil under a reflux condenser for thirty minutes, rotating the contents of the flask frequently during this period. Add 1 cc. of phenolphthalein indicator and titrate with 0.5 N hydrochloric acid.

A control or blank test should also be run, and the necessary corrections made in the following calculation.

3. **Calculation.**—The difference in the number of cc. of acid required for the fat and the blank is multiplied by 28.055 and divided by the weight, in grams, of fat taken for analysis; the resulting number is the saponification number.

C. Iodine Number.—The iodine number is the number of grams of iodine absorbed by 100 gm. of the fat or oil.

1. **Reagents.**—(a) *Iodine Bromide Solution (Hanus' solution).*—Dissolve 13.2 gm. of pure iodine in 1 liter of glacial acetic acid and add 3 cc. of bromine.

(b) *Potassium Iodide Solution:* 15 per cent aqueous solution.

(c) *Sodium Thiosulfate Solution:* 0.1 N.—Dissolve 24.820 gm. of sodium thiosulfate crystals, $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, in water and make up to 1 liter. One cc. of this solution equals 12.692 mg. of iodine.

(d) *Chloroform.*

(e) *Starch Indicator Solution.*

2. **Procedure.**—Into a 250-cc. glass-stoppered flask or bottle weigh accurately about 0.5 gm. of the fat, dissolve it in about 10 cc. of chloroform

and add exactly 25 cc. of iodine bromide solution from a buret. Stopper the flask or bottle securely and set aside for thirty minutes in a cool, dark place. The brown color of this mixture must persist during this period; if it does not, with the quantities given above, start a new test using a smaller weight of the fat. Add 10 cc. of potassium iodide solution and 100 cc. of water and titrate with 0.1 N sodium thiosulfate solution until the color of the free iodine is just discharged. Some prefer to use starch solution to give a sharp end point; if it is used, it must not be added to the solution until the color of the free iodine is nearly discharged. Starch strikes a blue color with free iodine and, when used as an indicator, the end point is the discharge of this color.

A blank test should be run with each test or group of tests, and the result of this blank used to correct the test made with those reagents.

3. **Calculation.**—The difference between the number of cc. of 0.1 N sodium thiosulfate solution required in the titration of the fat, and the number used in the blank test, is multiplied by 1.2692 and divided by the weight, in grams, of the fat examined. This is the iodine number.

D. **Hehner Number.**—The Hehner number is the percentage of insoluble fatty acids obtained from a fat or oil.

1. **Reagents.**—(a) *Potassium Hydroxide*: 1 to 1 aqueous solution.

(b) *Ethyl Alcohol*: 95 per cent.

(c) *Hydrochloric Acid*: specific gravity 1.12.

2. **Procedure.**—Weigh 2 to 3 gm. of the melted sample into a 500-cc. beaker, add 1 cc. of the potassium hydroxide solution and 20 cc. of 95 per cent ethyl alcohol. Cover the beaker with a watch glass and heat on a water bath until the liquid is clear and homogeneous. Evaporate off the alcohol and dissolve the soap formed in about 400 cc. of warm distilled water. Add 10 cc. of hydrochloric acid, and heat the beaker in the water bath almost to boiling until the clear oil floats. Dry and weigh a thick filter in a small covered beaker. Add the oil to the filter, and allow it to form a solid cake on top. Weigh the filter with the fat upon the weighed filter.

Remove the filter and wash the beaker with boiling water, then wash out the fat adhering to the beaker with boiling water, which is poured through the filter, taking care that the filter is never more than two-thirds full. If the filter paper is of good texture and wet beforehand, it will retain the fatty acids completely. If, however, oily particles are noticed in the filtrate, cool it by adding pieces of ice, remove the solidified particles with a glass rod and transfer to the filter. Cool the funnel by plunging it into cold water, remove the filter, place it in the weighing beaker, and dry it at 100° C. to constant weight.

3. **Calculation.**—The percentage of insoluble fatty acids, which includes the small amount of unsaponifiable matter, varies from 86.5 to 88 in butter fat; other fats and oils give values between 94.5 and 96.

E. **Refractive Index.**—Place a drop of melted fat on the under glass wedge of the refractometer, clamp and adjust the shadow to the center of the hair lines of the instrument. The scale reads the index of refraction.

TABLE 45.

	Refractive index	Butyro- refractometer reading
Natural butter	1 4590 to 1 4620	49 5 to 54.0
Oleomargarine	1 4650 to 1 4700	58 6 to 66 4
Mixtures	1 4620 to 1 4690	54 0 to 64.8

This physical property is very helpful in determining the purity of many substances.

Refractive indices and butyro-refractometer readings at 25° C. according to Wollny, are given in Table 45.

F. Microscopic Examination.—Place on a slide a small portion of the fresh, unmelted butter taken from the inside of the mass, add a drop of pure olive oil, apply a cover glass with gentle pressure, and examine with magnification of 120 to 150 diameters for crystals of lard, etc. Examine the specimen with polarized light and a selenite plate without the use of oil. Pure fresh butter will show neither crystals nor a parti-colored field with selenite. Renovated butter or other fats melted and cooled and mixed with butter will usually present crystals and variegated colors with the selenite plate.

For further microscopic study, dissolve in a test tube, 3 to 4 cc. of the melted fat in 15 cc. of ether. Close the tube with a loose plug of cotton and allow to stand for twelve to twenty-four hours at 20° to 25° C. When crystals form at the bottom of the tube, remove with a pipet, place on a slide, cover and examine under the microscope. The crystals formed by later deposits may be examined in a similar manner. Compare with crystals obtained in the same way from samples of known purity.

G. Foam Test.—Heat 2 to 3 gm. of the sample, in either a spoon or dish over a free flame. True butter will foam abundantly, whereas process butter will bump and sputter like hot grease, without foaming. Oleomargarine behaves like process butter but chemical tests will determine whether the sample is oleomargarine or butter.

XII. Rancidity.—The production of rancidity is due to two main causes, hydrolysis of the glycerides and oxidation of the unsaturated fatty acids.

Kerr's Modification of Kreis' test, one of the most satisfactory tests for rancidity, is as follows:

Shake vigorously 10 cc. of the melted fat for thirty seconds with 10 cc. of concentrated hydrochloric acid, specific gravity 1.19. Add 10 cc. of 0.1 per cent solution of phloroglucinol in ether and shake the mixture. If no red or pink color develops in the acid layer, the sample is passed as satisfactory; if a color develops, the sample is diluted with a non-reacting substance such as kerosene until no such color appears. A positive test in a dilution of 1 part of fat in 20 parts of kerosene would indicate a degree of rancidity evident to taste and smell.

MEAT AND MEAT PRODUCTS, ETC.

I. Preparation of Sample.—Fresh, dried and smoked meats are separated from bone and passed rapidly through a food chopper three times, mixing thoroughly after each grinding. The entire contents of a can in the case of canned meats are similarly ground. Remove the casings from sausages and grind in the same manner. For meat food products the "Waring Blender" is the easiest and the most convenient to use as it gives a homogeneous mass for analysis.

II. Added Water in Sausage, etc.—1. Moisture.—Weigh accurately about 10 gm. of the ground sample into a tared weighing-bottle, about 2 inches in diameter, containing a short glass rod flattened at one end. Remove 2 to 3 gm. for protein determination. Reweigh the remainder in

the bottle, spread it out in a thin layer over the sides and bottom of the bottle by means of the glass rod, and dry at atmospheric pressure at 101° to 102° C. for sixteen to eighteen hours, or at 125° C. (not lower than 120° C. nor more than 130° C.) for two to three hours to practically constant weight.

2. **Nitrogen.**—Determine as for Milk on page 291, using the portion removed in I, above. Calculate the protein by multiplying the total nitrogen by 6.25.

3. **Added Water.**—Multiply the percentage of protein calculated from the nitrogen determination by 4 and subtract the result from the percentage of moisture found. Report the difference, if any, as added water.

III. **Crude Fat in Sausage—Official.**—1. **Reagents.**—(a) *Ethyl Ether*, anhydrous.

(b) *Petroleum Ether.*—Boiling range 30° to 65° C.

2. **Procedure.**—Weigh 5 to 7 gm. of the prepared sample of meat into a previously weighed thimble and dry at 100° C. for at least two hours. After drying, the thimble is placed in the extractor of the Soxhlet extraction apparatus and to the flask are added 50 cc. of ethyl ether and 50 cc. of petroleum ether. Allow the extraction to proceed for sixteen hours after which time the flask will contain the ethers and the fat. The ethers are vaporized on a water bath and the flask is then placed in an oven (100° C.) for a period of one and a half hours. Cool in a desiccator and weigh. The fat is rinsed out with petroleum ether, the flask dried in an oven, cooled in a desiccator and weighed.

3. **Calculation.**—

$$\frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100 = \text{percentage of fat}$$

IV. **Determination of Salt in Meat.**—1. **Reagents.**—(a) *Silver Nitrate*—0.1 N solution.

(b) *Nitric Acid*—concentrated.

(c) *Potassium Permanganate*—5 per cent aqueous solution.

(d) *Ethyl Ether*.

(e) *Potassium Thiocyanate*—0.1 N solution.

(f) *Ferric Ammonium Sulfate*—saturated aqueous solution.

2. **Procedure.**—Weigh 2.5 to 3 gm. of the finely ground and thoroughly mixed sample into a 250-cc. flask and add 50 cc. of 0.1 N AgNO₃ solution, thoroughly wetting the meat. This should be added either by means of a buret or a pipet. Add 15 cc. of nitric acid, heat to boiling and allow to boil briskly until the meat is completely disintegrated (about ten minutes). Add potassium permanganate solution in successive small amounts by means of a pipet until the solution becomes colorless or nearly so. Add 25 cc. of water and boil for approximately fifteen minutes. Cool and dilute to a volume of about 150 cc. Add 25 cc. of ethyl ether to dissolve the separated fat and shake. Add 1 cc. of ferric ammonium sulfate indicator and titrate with 0.1 N potassium thiocyanate until the first brownish color change is noted that persists for thirty seconds.

3. **Calculation.**—Subtract the number of cc. of potassium thiocyanate from 50, the cc. of AgNO₃ used. The remainder multiplied by 0.00585 and by 100 divided by the weight of the sample gives the percentage of salt.

V. Starch in Sausage, etc. A. Qualitative Test—Tentative.—Treat 5 to 6 gm. of the sample with boiling water for two to three minutes, cool the mixture, and test the supernatant fluid with an iodine solution prepared by dissolving 0.5 gm. of iodine and 2 gm. of potassium iodide in 150 cc. of water.

A small quantity of starch may be present from the use of spices, but a marked reaction indicates that starch or flour has been added. Microscopic examination will disclose the variety.

B. Quantitative Method (McVey).—1. Reagents.—(a) *Potassium Hydroxide Solution*—8 per cent solution of KOH in 95 per cent alcohol.

(b) *Hydrochloric Acid Solution*—5.7 N.

2. Procedure.—Using a balance sensitive to 0.1 gm., weigh 10 gm. of the comminuted and mixed meat into a 150-cc. beaker. Add 50 cc. of KOH solution, break up lumps with a stirring rod, and heat on a steam bath for thirty to forty-five minutes, or until the product has been completely disintegrated. Dilute with 40 to 50 cc. of 95 per cent ethyl alcohol, and add with stirring, about 1 gm. of "filter-cel," of a grade unaffected either by dilute HCl or dilute KOH solution. After a few minutes, filter with the aid of suction through a Gooch crucible fitted with a disk of filter paper, wash thoroughly with 95 per cent ethyl alcohol, and suck as dry as possible. Holding the crucible and contents inverted over the same beaker used for the digestion, dislodge the contents by tapping the sides of the crucible. Remove any material remaining in the crucible by means of a small piece of filter paper, and add the paper to the material in the beaker.

Add 5.7 N HCl in small portions to the cereal-filter-cel mixture in the beaker while stirring or kneading the mass with a stirring rod until the contents assume a creamy consistency. Transfer the contents, together with the fragments of filter paper, quantitatively to a 100-cc. volumetric flask with the aid of a jet of 5.7 N HCl from a small wash bottle, and add more of the HCl if necessary to make a volume of 50 to 60 cc. in the flask.

In another Gooch crucible prepare a moderately thin asbestos mat, fill the crucible about two-thirds full of filter-cel, dry at 100° C., and when cool tare on an analytical balance. By means of a graduate measure 115 cc. of 95 per cent ethyl alcohol into a 250-cc. beaker. Pipet a 50-cc. aliquot of the filtered starch solution into the alcohol and stir vigorously. Then before the precipitated starch has coagulated or settled, pour the filter-cel from the tared crucible into the alcohol-starch mixture and again stir thoroughly. Allow to settle for about five minutes, then using suction filter through the tared Gooch crucible, wash thoroughly with 95 per cent alcohol, using a rubber-tipped stirring rod, if necessary, to dislodge all starch material from the sides of the beaker. Dry overnight at 100° C., or for one and a half hours at 125° C., cool in a desiccator, and weigh immediately on removal from the desiccator, as the material is hygroscopic.

3. Calculation.—Multiply the weight of starch found by 20 to obtain the percentage of starch in the sample. To obtain the percentage of cereal, multiply the per cent of starch found by the appropriate factor (the

reciprocal of the proportion of starch in the particular cereal product used), which in the case of corn flour is 1.27.

VI. Determination of Nitrates—Tentative—Xylenol Method.—1. Apparatus and Reagents.—Use a simple distilling apparatus including a distillation bulb. A glass condenser of a type utilizing a rapidly moving film of water as a cooling medium is recommended. Quickly remove any nitroxylenol solidifying in the condenser by stopping the flow of water and allowing the condenser to become warm.

(a) *Meta-xylenol (1-hydroxy, 2, 4-dimethylbenzene)*. (Eastman's preparation No. 1150 or equivalent.)

(b) *Silver Ammonium Hydroxide*.—Dissolve 5 gm. of nitrate-free Ag_2SO_4 in 60 cc. of NH_4OH . Heat to boiling, concentrate to about 30 cc., cool, dilute to 100 cc. with H_2O .

(c) *Bromocresol Green Indicator*.—Dissolve 0.1 gm. of bromocresol green in 1.5 cc. of 0.1 N NaOH and make up to 100 cc. with H_2O .

(d) *Standard Nitrate Solution*.—Dissolve 0.1804 gm. of recrystallized KNO_3 in H_2O and make up to 1 liter, or dilute 17.85 cc. of 0.1 N HNO_3 to 1 liter. Ten cc. contain 0.25 mg. of nitrate nitrogen.

(e) *Nitrate-free Water*.—Add several crystals of KMnO_4 and several pellets of NaOH to distilled water and redistill.

2. Procedure.—Mix 5 to 10 gm. of finely comminuted and thoroughly mixed sample with 80 cc. of warm H_2O . Break up all lumps and heat on the steam bath for one hour, stirring occasionally. Transfer to a 100-cc. volumetric flask, cool, make up to the mark, and mix. Filter or allow to settle, and pipet 40 cc. of filtrate, or supernatant liquid, into a 50-cc. volumetric flask. Add 3 drops of bromocresol green indicator. Add H_2SO_4 (1 + 10) dropwise until the color changes to yellow. Oxidize nitrites to nitrates by adding 0.2 N KMnO_4 solution dropwise with shaking until a faint pink color remains fifteen to thirty seconds. Add 1 cc. of H_2SO_4 (1 + 10) and 1 cc. of phosphotungstic acid solution (20 gm. in 100 cc.). Make up to the mark, mix and filter.

Measure into a 500-cc. Erlenmeyer flask an aliquot (not more than 20 cc.) containing from 0.005 to 0.15 mg. of nitrate nitrogen. If more than 20 cc. are required, make slightly alkaline and concentrate by evaporation. Add a sufficient quantity of silver ammonium hydroxide solution to precipitate all chlorides and most of the excess phosphotungstic acid. A slight excess of silver reagent is not harmful; 1 or 2 cc. are usually sufficient. Without decanting or filtering, add a volume of H_2SO_4 (3 + 1) approximately three times the volume of the liquid in the flask. Stopper, mix, cool to about 35°C ., add 0.05 cc. (1 to 2 drops) of m-xylenol, stopper, shake, and hold at 30° to 40°C . for thirty minutes. A yellow to brownish color, indicative of nitrates, will appear. A bright red precipitate, due to incomplete removal of phosphotungstic acid, may also appear.

After nitration is complete, add 150 cc. of H_2O , taking care to wash off the stopper and distil until 40 to 50 cc. have passed over into the receiver containing 5 cc. of NaOH (10 gm. per liter). Transfer the distillate to a 100-cc. volumetric flask, make up to volume with H_2O , and determine the nitrate nitrogen by comparing the color of a suitable aliquot with a set of graded color standards containing 0.003–0.006 mg. of nitrate nitrogen.

Prepare the color standards from 1, 2 and 3 cc. respectively of the standard nitrate solution. Treat each one in the same manner as the

unknown, beginning with "without decanting or filtering add a volume of H_2SO_4 (3 + 1), etc., etc.," using 30 cc. of H_2SO_4 (3 + 1) and 0.05 cc. of the meta-xylene. Make each distillate up to 100 cc. Prepare fresh color standards each day since they become cloudy on standing.

Compare the unknown with the standards using Nessler tubes. If it is necessary to dilute the unknown to obtain a color match, the dilution should be made with a solution prepared exactly as the standards are, using water instead of the standard nitrate solution.

3. Calculation.—Calculate the amount of nitrate present in parts per million.

VII. Determination of Nitrites (Applicable to Cured Meat)—Tentative.

—1. Reagents.—(a) *Mercuric Chloride Solution*—saturated solution.

(b) *Sulfanilic Acid Solution*.—Dissolve 1 gm. of sulfanilic acid in hot water, cool, and dilute to 100 cc.

(c) *Alpha-naphthylamine Hydrochloride Solution*.—Boil 0.5 gm. of the salt with 100 cc. of H_2O , kept at constant volume, for ten minutes.

(d) *Standard Nitrite Solution*.—Dissolve 1.1 gm. of AgNO_2 in NO_2 -free water, precipitate Ag with NaCl solution, dilute to 1 liter, mix and allow to settle. Dilute 100 cc. to 1 liter and then 10 cc. of this solution to 1 liter, using in each case NO_2 -free water. One cc. of the last solution = 0.0001 mg. of nitrogen or 0.0005 mg. of NaNO_2 .

(e) *Nitrite-free Water*.—Add several crystals of KMnO_4 and several pellets of NaOH to distilled water and redistil.

2. Procedure.—Weigh 5 gm. of finely comminuted and thoroughly mixed sample into a 50-cc. beaker. Add about 40 cc. of nitrite-free H_2O heated to 80°C . Mix thoroughly by stirring with a glass rod, taking care to break up all lumps, and transfer to a 500-cc. graduated flask. Wash out the beaker and rod thoroughly with successive portions of hot H_2O , adding all washings to the flask. Add sufficient hot water to bring the contents of the flask to a volume of about 300 cc., and place the flask on a steam bath. Let stand for two hours, shaking occasionally. Add 5 cc. HgCl_2 solution and mix. Cool to room temperature, make up to the mark with nitrite-free water, and mix again.

Place 100 cc. of sample in a 100-cc. Nessler tube and add HCl dropwise until the sample shows an acid reaction to litmus. Add 1 cc. of sulfanilic acid and 1 cc. of the alpha-naphthylamine hydrochloride solution, and thoroughly mix. Set aside for thirty minutes with other Nessler tubes containing known quantities of the standard nitrite solution made up to 100 cc. with NO_2 -free H_2O and acidified with HCl, treated with the sulfanilic acid and the alpha-naphthylamine hydrochloride solutions in the same manner as the sample.

3. Calculation.—Determine the quantity of N by comparing the depth of pink color in known and unknown and report results as parts of NaNO_2 per million.

VIII. Detection of Soybean in Meats in the Presence of Cereal (McVey—unpublished).—1. Reagents.—(a) *Alcoholic Potassium Hydroxide*.—Dissolve 8 gm. of KOH in 92 gm. of 95 per cent alcohol.

(b) *Ethyl Alcohol*—25 per cent.

2. Procedure.—Treat 10 gm. of finely ground meat with alcoholic KOH and heat on a water bath until the meat has disintegrated; usually forty-five minutes are sufficient. Transfer the liquid and residue to a 100-cc.

graduated sedimentation tube, make up to 100 cc. with 95 per cent ethyl alcohol, and allow to settle. Decant off the supernatant liquid as completely as possible and cover the residue with 40 to 50 cc. of warm water. Stopper the tube and shake vigorously, allow to stand for a few minutes until the foam subsides, then transfer to a 50-cc. centrifuge tube, and centrifugalize for five minutes. Pour off and discard the supernatant liquid, stopper and shake, or mix the residue with 15 cc. of 25 per cent ethyl alcohol, and centrifugalize for five minutes. Pour off the

supernatant liquid and examine the residue under the microscope for the characteristic "hour-glass" cells, preferably with the use of polarized light.

IX. The Detection of Dried Skim Milk in Meat Products.—As meat and meat products normally do not contain lactose, the lactose added in the form of dried skim milk may be detected by the formation of lactosazone, the crystals being easily recognizable under the microscope. Lactosazone crystallizes in "hedgehog" clumps with projecting spines, which usually, although not always, terminate in long hair-like appendages.

1. Reagents.—(a) *Alumina Cream.*—Prepare a cold saturated solution of alum in H_2O . Add NH_4OH with constant stirring until the solution is alkaline to litmus, allow the precipitate to settle, and wash by decantation with H_2O until the wash water gives only a slight test for sulfate with $BaCl_2$ solution. Pour off the excess of H_2O and store the residual cream in a stoppered bottle.

(b) *Glacial Acetic Acid.*

(c) *Phenylhydrazine Hydrochloride.*

(d) *Sodium Acetate.*

tion with the aid of a separatory funnel. To 25 to 30 cc. of the aqueous solution add 10 cc. of alumina cream, shake, allow to stand a few minutes, and filter through a wet filter paper. Add 25 cc. of the filtrate to 1 gm. of good adsorbent charcoal in a 125-cc. Erlenmeyer flask, shake vigorously, boil a few seconds, cool thoroughly, and allow to stand ten minutes, with frequent shaking. Filter with suction through a Gooch crucible containing a disk of filter paper. Wash with 2 to 3 cc. of water and suck dry. Transfer the charcoal from the crucible to a 125-cc. Erlenmeyer flask, add 10 cc. of water and 1 cc. of glacial acetic acid, and boil for about ten minutes. Filter the hot mixture through a small filter paper into a large test tube containing 0.5 gm. of pure phenylhydrazine hydrochloride with 2 gm. of sodium acetate. Place tube in a boiling water bath, and, after a few minutes, mix by shaking, and heat for forty-five minutes. Remove the tube from bath, filter while hot, and allow to stand at room temperature at least one hour. If no crystals have formed after one hour, stopper the tube and allow to stand until crystals form. The test may be considered negative if no lactosazone crystals form after standing eighteen hours. Pipet off a little of the deposit, if any, on to a microscope slide, cover with a cover glass and examine under magnification of 100 to 500 diameters.

X. Preservatives—Official.—1. **Formaldehyde.**—Macerate 200 to 300 gm. of sample with 100 cc. of water in a mortar. Transfer to a 500- to

800-cc. short-necked, pyrex distilling flask, make distinctly acid with phosphoric acid, connect with a condenser, and distil 40 to 50 cc.

Proceed with this distillate as on page 295.

2. *Salicylic Acid*.—Grind the meat sample and mix thoroughly. Transfer 50 to 200 gm. to a 500-cc. flask, add water to make a volume of about 400 cc., and shake until the mixture is uniform. Add 2 to 5 gm. of calcium chloride and shake until dissolved. Make distinctly alkaline to litmus with 10 per cent NaOH solution. Shake thoroughly at intervals over a period of two hours, and then filter.

Using the filtrate, or a part thereof, proceed as on page 295, with ether extraction after acidifying the solution by adding one-tenth its volume of hydrochloric acid, 1 part of concentrated acid to 3 parts of water.

3. *Benzoic Acid*.—Using the filtrate as prepared above, proceed as on page 295.

4. *Boric Acid and Borates*.—Heat the ground sample with sufficient water to make a fluid mass, acidify with hydrochloric acid, using 7 cc. of concentrated acid to each 100 cc. of sample and proceed as on page 296.

LARD

The determination of the Reichert-Meißl-Polenske-Kirschner values, saponification number, iodine number, Helmer number, refractive index and the microscopic examination may be made in the same manner as given for Butter, pages 303 to 307.

In addition employ the following procedure in checking possible adulteration:

1. *Detection of Foreign Fats Containing Tristearin*.—1. *Reagents*.—(a) *Acetone*: C.P.

(b) *Ether*: Ethyl.

(c) *Potassium Hydroxide*: 0.5 N alcoholic solution.

2. *Procedure*.—Weigh 5 gm. of the melted lard into a 25-cc. glass-stoppered cylinder and add warm acetone to the 25-cc. mark. Shake until thoroughly mixed and allow to stand for eighteen hours at a temperature of 30° C. Carefully decant the acetone from the mass of crystallized glycerides, which are usually found in a firm mass at the bottom of the cylinder. Add warm acetone in three portions of 5 cc. each from a small wash-bottle, taking care not to break up the deposit while washing, and decant the first two washings. Shake the third portion in the cylinder, and by a quick movement transfer with the crystals to a small filter paper. Wash the crystals with five successive small portions of warm acetone and remove the excess acetone by suction. Spread the paper with the crystals on a clean dry glass plate and break up the lumps by gentle pressure with a spatula. When dry, thoroughly pulverize the mass and determine the melting point in a sealed 1-mm. capillary tube. A melting point below 63° C. is regarded as evidence of adulteration; below 63.4° C. is suspicious.

Transfer the mass of crystals to a 50-cc. beaker, add 25 cc. of 0.5 N alcoholic KOH and heat on a steam bath until saponification is complete. Pour the solution into a separatory funnel containing 200 cc. of water, acidify, add 75 cc. of ether and shake. Draw off the acid layer and wash the ether solution at least three times with water. Transfer the ether solution to a clean dry 50-cc. beaker and evaporate on a water bath. Dry the fatty acids at 100° C. for two hours; then determine the melting point.

If the melting point of the glycerides, plus twice the difference between the melting point of the glycerides and the melting point of the fatty acids, is less than 73°C ., the lard is regarded as adulterated.

SALAD DRESSINGS

Before removing any portion of the sample for analysis and each time a subsequent portion is removed, mix until it is homogeneous.

I. **Total Solids.**—Weigh 10 gm. of the sample into a previously weighed dish containing 10 to 15 gm. of clean dry quartz sand, weighed with the dish. Evaporate to apparent constant weight at the temperature to exceed 100 mm. of mercury.

on samples high in solids should be made at hourly intervals.

II. **Total Acids.**—Dilute 10 gm. of the sample with 400 to 500 cc. of recently boiled and cooled water and titrate with 0.1 N alkali, using phenolphthalein indicator. Calculate as acetic acid. One cc. of 0.1 N alkali equals 0.006 gm. of acetic acid.

III. **Oil.**—Weigh 2 gm. of the sample into a Rohrig tube or similar apparatus, add 2 cc. of 28 per cent ammonium hydroxide, and mix thoroughly. Add 10 cc. of 95 per cent alcohol and again mix. Then add enough water to fill the tube to the level of the outlet. Add 25 cc. of ether, shake the tube vigorously for thirty seconds, add 25 cc. of petroleum ether, boiling point below 65°C ., and shake again for thirty seconds. Let stand for twenty minutes, or until the upper liquid is practically clear. Draw off as much as possible of the ether-oil solution into a flask through a small quick-acting filter. Again extract the liquid in the tube, using 15 cc. of each ether. Repeat the extraction a third and fourth time, using 15 cc. of each ether at a time. Add a glass bead and evaporate the ether solution slowly on a steam bath, then dry the oil to constant weight at 70°C . under a pressure not exceeding 100 mm. of mercury. After weighing the flask, bead and oil, clean the flask and bead thoroughly with petroleum ether, dry and weigh. Deduct this weight from that of flask, bead and oil to obtain the weight of the oil.

The oil obtained may be subjected to the identifying tests given under Butter, pages 303 to 307.

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CHAPTER XIX

TOXICOLOGICAL METHODS

By CLEON J. GENTZKOW, WALTER C. TOBIE and LEO R. GOLDBAUM

TOXICOLOGICAL analysis, especially of body fluids and tissues, is work for an expert, and should never be undertaken except by those having special training and experience, since the life and liberty of others may depend upon the findings. The rough qualitative tests sometimes attempted by persons with little or no experience in toxicology, in cases of suspected poisoning, is *most unwise*, since it uses up irreplaceable material and may lead to a miscarriage of justice.

The only exception to the above rule occurs in cases where the victim of suspected poisoning is still alive, so that definite findings would be of value in clinical treatment. In such cases, simple tests for suspected poisons may be done on the urine, vomitus, stomach washings, suspected food and drink, suspected drugs, etc., to determine the nature of the poison, if any. The findings should be regarded as indicative only, and most of the suspected material should be sent as soon as possible to a toxicological laboratory for confirmatory testing.

The purpose of this chapter is two-fold: (a) To provide an *introduction* to toxicology for those who may be called upon to do simple toxicological tests in the absence of a professional toxicologist, and (b) To provide *general* information for clinicians, pathologists, technicians, and others who may be called upon to investigate cases of suspected poisoning and to collect and pack specimens for shipment to a toxicological laboratory.

Owing to limitations of space, it will be possible to mention only the commoner toxic agents. The analytical methods are necessarily condensed and simplified so as to be of *general* usefulness, and may have to be modified to suit special cases. They should not be followed slavishly or carried out in a routine fashion, since nearly every case of suspected poisoning requires individual handling differing slightly from all others. In toxicology, as in other fields, there is no substitute for the trained judgment which is the result of experience. For special methods, and unusual poisons, see the references at the end of the chapter. It is not intended that the present chapter should be regarded as the final and ultimate authority on toxicological questions arising in the Army.

The descriptions of the duties of the clinician, pathologist, and toxicologist which follow, overlap to some extent. Since in some cases, a single individual may function in two or more of these capacities, it is desirable that those concerned in toxicological cases should be familiar with all the duties outlined.

DUTIES OF THE CLINICIAN

The first duty of the clinician is to save life and relieve suffering, by the removal of the poisons, administration of chemical antidotes, and treatment of the symptoms. The second duty is to aid in detecting the poisons involved and in fixing the responsibility on the proper person. Therefore,

the clinician must carefully observe the symptoms, and record carefully in permanent form all pertinent aspects of the case, especially those which appear to indicate or to rule out the possibility of poisoning. If the victim is still alive, a statement should be obtained from him if possible. All urine, feces, vomitus, stomach washings, and suspected food, drink, or drugs, should be saved for examination. A careful search should be made of the victim's effects and surroundings for drugs, medicaments, etc., which may have served as the toxic agent. Secure all materials, regardless of the labels, since substitutions may have been made, either deliberately or through carelessness. Secure also any emptied containers, such as bottles, drinking glasses, etc., since it is sometimes possible to identify very small residual amounts of poisons remaining on them. In many cases of poisoning, the emptied container from which the poison was

TABLE 46

VOMITING: Frequently associated with purging and abdominal pain.

Poisons: Arsenic, antimony, corrosive acids and alkalis, barium, cantharides, digitalis, copper, iodine, mercury, phosphorus, phenols, alcohols, zinc, poisoned foods.

Diseases: Gastritis and enteritis, gastric and duodenal ulcer, cholera, uremia, acidosis, early pregnancy, brain tumor, onset of many infectious diseases.

CONVULSIONS:

Poisons: Brucine, camphor, cyanides, strychnine, etc.

Diseases: Uremia, eclampsia, tetanus, epilepsy, many acute diseases of the cerebrospinal system, especially meningitis.

COMA:

Poisons: Opium and its alkaloids and derivatives, chloral, sulfonal, trional, tetronal, barbiturates, such as amytal, phenobarbital, nembutal, etc., paraldehyde, chloroform, cyanides, CO, CO₂, atropine, hyoscine, scopolamine, large doses of alcohol, etc.

Diseases: Uremia, eclampsia, acidosis, cerebral hemorrhage, cerebral embolism and thrombosis, brain injuries, epilepsy, and other brain diseases.

DILATATION OF PUPIL:

Poisons: Belladonna and its derivatives, hyoscyamine, stramonium, gelsemium, cocaine, nicotine.

Diseases: Those causing optic atrophy, sympathetic irritation, or oculomotor paresis.

CONTRACTION OF PUPIL:

Poisons: Derivatives of opium, physostigmine, pilocarpine, muscarine.

Diseases: Tabes and some other diseases of the central nervous system.

GENERAL OR PARTIAL PARALYSES:

Poisons: Cyanides, CO, CO₂.

Diseases: Apoplexy, brain tumor, botulism, meningitis.

SLOW RESPIRATION:

Poisons: Opium and its derivatives, CO, hypnotics.

Diseases: Uremia, compression of the brain from any cause.

RAPID RESPIRATION:

Poisons: Atropine, cocaine, CO₂, etc.

Diseases: Acute respiratory diseases, lesions of medulla, hysteria.

DELIRIUM:

Poisons: Atropine, cocaine, cannabis (hashish or marijuana).

Diseases: Epilepsy, insanity, delirium tremens, organic brain diseases such as meningitis, encephalitis, etc., nephritis, etc.

DYSPNEA:

Poisons: Strychnine (during the convulsions), cyanides, CO.

Diseases: Those of cardiac and respiratory systems, lesions of medulla and vagus.

CYANOSIS:

Poisons: Nitrobenzene, aniline, acetanilide, opium.

Diseases: Same as dyspnea, prolonged convulsions due to any cause.

taken may be found at a *considerable distance* from the victim. In other cases the container is never found, having been hidden or destroyed. Lock up all specimens and suspicious materials in a safe place to prevent possible tampering until such time as they can be analyzed. Very often a tentative identification of pills, tablets, capsules, etc., may be made by consulting with a well-qualified pharmacist.

All available information should be furnished, whether it is considered important or not, first, to the pathologist in case of death and autopsy, and second, to the toxicologist. *Too much information is better than too little*, since apparently trivial facts may furnish important clues. It is rarely possible to determine a poison on clinical symptoms alone, or even on postmortem findings, but the indications obtained may be of inestimable value to the analyst. Suggestions may be offered that search be made for some particular poison to which the symptoms point, but furnish complete data to the analyst nevertheless, and let him deduce from such facts the probable type of poison implicated.

Many diseases produce symptoms which are identical with those caused by poisons and differentiation may be difficult until a careful study has been made. Table 46 lists symptoms which are common to both disease and poisoning, together with the diseases and poisons causing them, as an aid in differential diagnosis. However, it must be remembered that unusual symptoms may be produced in an individual by the various poisons, just as unusual symptoms may occur in any disease.

Poisoning due to bacterial contamination of food is far more common than that due to chemical substances accidentally or intentionally introduced. Accordingly, an epidemiological and bacteriological investigation should be made whenever a number of cases of sickness or death occur after eating the same food. For methods see "Bacterial Food Poisoning," page 526. Non-fatal chemical food poisoning may be caused by metals dissolved out of containers used for food. Severe chemical food poisoning, fatal and non-fatal, sometimes arises from the accidental or deliberate introduction of rat or roach poisons such as sodium fluoride, arsenic, barium salts, thallium salts, or white phosphorus pastes, into food. Chemical poisoning usually comes on *rapidly*, almost immediately after eating the food; bacterial food poisoning is usually not immediately apparent and is often due to food eaten as long as six to thirty-six hours previously. This must be kept in mind in selecting samples for analysis.

DUTIES OF THE PATHOLOGIST

In all fatal cases of suspected poisoning, a complete autopsy is necessary, not only to determine if lesions due to poisons are present, but also to *rule out natural causes of death*. It is assumed that the pathologist is familiar with the general technic of postmortem examinations, so that stress need be laid only on the modifications required in toxicological cases.

The usual thorough inspection of the body should be made even more detailed and searching if poisoning is suspected. Careful examination of lips, tongue, and mucosa of the mouth and throat should be made for evidence of corrosive action. The condition of the pupils, extent and degree of rigor, and presence and character of odors should be noted. Search should be made for puncture marks of hypodermic needles, and if

found, the tissues in the immediate area dissected out for chemical examination.

It is advisable to open the abdomen first, and to observe the organs *in situ* before opening the chest, in order to prevent the admixture of blood and pleural fluid. If the evisceration method is used, be sure to tie off the esophagus and to secure the required blood and urine samples before removal of the viscera *en bloc*.

The most important point to remember is to secure LARGE amounts of APPROPRIATE material for toxicological examination. The most frequent error made in toxicological cases is to remove only the stomach and its contents for chemical examination. It should be obvious that if an individual lives for several hours or days after taking a poison by mouth, little if any of the poison will remain in the stomach, particularly if vomiting occurs. If death occurs *suddenly* after taking food, drink, or drugs by

be sufficient, but it is other materials as well. amounts of tissue or body fluids taken more or less at random, but such specimens are *not* suitable for any worthwhile toxicological study. If the deceased lived for several days after poisoning, only cumulative poisons will be readily detectable, although histopathological lesions will often be indicative of the poison involved.

Before beginning the autopsy, there must be on hand an adequate number of clean containers, large enough to take generous specimens. Glass-topped Mason jars of the spring type and wide-mouthed glass-stoppered bottles are the most suitable. Other types of containers should be used only if nothing better is available. Old chemical bottles should not be used, owing to the possibility of introducing contaminations, but the wide-mouthed bottles used for dehydrated culture media are fairly satisfactory. Narrow-mouthed bottles with firmly fitting glass stoppers are better containers for liquids than are test tubes. The latter if used, must be very well packed to avoid breakage in shipment.

Whenever possible, *send in all the urine* even if only a few cc. are available. Since the kidneys secrete practically all soluble poisons or their metabolic products, an examination of the urine alone will frequently reveal the poison involved. It should be withdrawn from the bladder before removal of the viscera, using a clean catheter, or a glass syringe and needle.

Prior to use all containers must be thoroughly cleaned. The best method is to scrub thoroughly with soap and water, rinse, immerse in cleaning solution (see page 355) for several hours, rinse with at least ten to twelve changes of hot water, and finally with two or three changes of distilled water. It will aid greatly if a supply of clean containers is prepared in advance and reserved for the taking of toxicological specimens when the occasion arises.

If at all possible, the autopsy should be done prior to embalming, since embalming fluids have been prohibited by law in most states, owing to their interference in toxicological analyses for arsenic, but may still be

encountered in a few cases. Similarly, specimens *must not* be allowed to become contaminated with germicides or antiseptics in the course of the autopsy.

Table 47 indicates the minimum amounts of material which should be taken for toxicological analyses. No definite rules can be laid down, since poisons vary in the ease with which they are detected, and in the amounts present in different cases. However, it is well to send in just as much material as possible. *Too much material is better than too little.* The stomach and contents should be put in one jar, intestinal contents in another jar. A single container may be used for the solid viscera.

TABLE 47

<i>Specimen</i>	<i>Minimum amount</i>	<i>Poison for which specimen is best suited</i>
Urine	All available	Valuable in nearly all types of poisoning
Stomach contents	All available	For cases in which poison is known or thought to have been taken by mouth within a few hours.
Intestinal contents	About 100 grams	For cases in which poison was taken by mouth within one or two days.
Blood	About 10 cc.	All gaseous poisons. To determine methemoglobin, carboxyhemoglobin, sulfonamides, bromides, etc. Drowning (see page 345)
Brain	500 grams	Barbiturates, alkaloids, volatile poisons, acute alcoholism.
Liver	500 grams	Metals, barbiturates, fluorides, oxalates, sulfonal, and many other poisons.
Kidney	One kidney	Metals, especially mercury, sulfonamides
Bone	200 grams	Lead, arsenic, radium (especially chronic poisonings).
Lung	One lung	For inhaled poisons, and to prove if poisons entered by inhalation.
Hair	As much as possible	Chronic (not acute) arsenic poisoning
Muscle	200 grams	In most acute poisonings, and cases in which internal organs are badly putrefied.
Spinal fluid	All available	Acute alcoholism and similar types of poisoning.

In cases of suspected drowning, take 6 cc. or more blood from the right and left sides of the heart, using pipets with relatively large openings and being careful not to perforate the septum. Label the bottles "left heart" and "right heart." Take a specimen of the water, 25 cc. or more, in which the person supposedly drowned. By chloride analysis of the three specimens, drowning can often be proved or disproved, since water entering the lungs diffuses into the blood and reaches the left heart before the right heart. The increase in chloride in the left heart blood in salt water drowning is more pronounced than the decrease which occurs in fresh water drowning. The test is of value only if the foramen ovale of the heart is normally closed.

The autopsy protocol furnished to the medical jurist should be complete and detailed, with special attention paid to any findings which tend to confirm or rule out poisoning. For instance, a typical pinkish-red stain on the skin suggests carbon monoxide, a typical "bull's-eye lesion" suggests mercury poisoning. These leads are particularly valuable in indicating which poisons should be tested for first.

As soon as a specimen is placed in a container, label the latter with *all* the information required to identify the specimen, such as full name, rank and serial number of the individual, name of the specimen, date, and station at which the autopsy was done. Poorly labeled specimens cause much confusion when received for analysis.

Usually it will not be possible to deliver the specimens directly to the toxicologist. Therefore some preservative must be used. The best method is to refrigerate with solid carbon dioxide ("dry ice"), which may be secured at most ice cream and packing plants, or other commercial sources of supply. Pack the jars in a heavy cardboard container with *generous* amounts of dry ice in paper bags on top of the specimens, and seal the package with strips of gummed paper. Packing must be done in such a way that the containers will remain in place even if all the dry ice evaporates. Specimens can be kept in perfect condition for seventy-two hours with dry ice. If ordinary ice is used, ship by express, making arrangements for re-icing en route.

When a chemical preservative must be employed, use about 100 cc. of ethyl alcohol, U.S.P. 95 per cent for each 100 gm. of sample. Send along about 250 cc. of the alcohol to be tested for possible extraneous substances. Ethyl alcohol must not be used as a preservative when one of the poisons in question is ethyl or any other alcohol. Do not use denatured alcohol, rubbing alcohol, or similar preparations, since the denaturants will give false and misleading results in analysis.

Formalin (formaldehyde) is *extremely undesirable* as a preservative of specimens for *toxicological* examination, since it will seriously interfere with the tests for most organic poisons. *Separate* specimens should be fixed in 10 per cent formalin for histopathological examination, since death may have been due to causes other than poisoning.

Handle specimens from living cases in the same manner. Above all, be generous with the samples. Send *all* of the vomitus, stomach washings, feces, urine, suspected food and drink, etc., and give the toxicologist *all available* information about the case.

Wrap each container in heavy paper, tie it up or seal it with cellulose tape or surgeon's tape, then seal top, bottom, and free edge, as well as the knot if string is used, with sealing wax. Mark the hot wax with some distinctive device such as a signet ring, or a coin having a scratch made across its face so that tampering would be evident immediately. Keep all specimens so prepared in your immediate possession or safely locked up until they are shipped to the toxicologist. The "*continuity of possession*" must not be broken. The material will become valueless for medico-legal purposes if any break in the record of its custody can be proved. Therefore, the precautions on safeguarding and sealing are *very important* in any case in which there is the least suspicion of foul play.

Those charged with investigating an unexplained death or suspected poisoning, should *continue* their investigation while awaiting the laboratory reports. The common tendency to merely send in specimens for laboratory examination and to then lapse into inactivity while awaiting the report, must be avoided. Much valuable information is frequently lost by this dilatory form of procedure.

DUTIES OF THE TOXICOLOGIST

Under ideal conditions, the toxicologist would be present at the autopsy, and would collect any specimens required on the basis of the clinical and pathological indications of the possible poison present. Under army conditions, this will rarely be possible, so that the toxicologist must work with the specimens and information furnished him. This makes it doubly

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toms and
autopsy protocol. For instance, chocolate-colored blood is suggestive of oxidizing agents such as chlorates, or perhaps nitrobenzene. The clinical symptoms (see Table 46) are often suggestive. If it appears necessary, the toxicologist endeavors to obtain supplementary information on the case.

He then systematicall
to the poisons suggested
other possible poisons are
the possibility of anyone tampering with the material which he is examining, by securing it at all times. He also takes scrupulous care to protect it from accidental contaminations. He keeps complete and careful notes. When the analyses are finished, the results are reported promptly and fully, in a written report, taking care that no significant aspects are neglected. Negative findings are as important as positive ones, and should be reported adequately.

OUTLINE OF TOXICOLOGICAL ANALYSIS

I. General Considerations.—Since the material available is always limited in amount, and cannot be replaced, it must be handled in such a way as to obtain the maximum amount of information from it. No general scheme of analysis can be laid down which is applicable to all cases. In the simplest case, it may be necessary merely to determine the presence or absence of a single suspected poison. In other cases, the detection and determination of two or more drugs having similar effects may be required. Thus in some cases of stupor or narcosis it may be necessary to test for barbiturates, and opium alkaloids. A search for a wide variety of poisons must be made inadequate,

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should be set aside for a quick determination. If a poison is
detected by a preliminary test, it should be subjected to one or more confirmatory tests, followed in most cases by a quantitative determination. The urine can be used for quick preliminary tests or reserved for confirmatory testing. Quantitative determinations are required in many cases, because the *quantity* of poison found together with its distribution in different organs, is of prime importance. The mere presence of a toxic substance is not necessarily proof that it was absorbed or ingested in a toxic form, or was a factor in causing the death. For example, in examining the gastro-intestinal contents, mercury may be present in the form of

calomel, barium as barium sulfate administered in connection with an X-ray examination, bismuth from bismuth subnitrate taken in a diarrhea mixture, etc. Similarly a finding of arsenic, mercury, or bismuth in the internal organs may merely indicate that they had been administered medicinally. By a determination of the *quantities* present, it is usually possible to obtain a fair idea of whether the substance can actually be implicated in the death. The toxicological findings must be correlated with the circumstances of the case, the clinical history, and the autopsy findings including histopathological examination, before one can arrive at definite conclusions.

In order to extract the largest possible amounts of poisons from the solid organs, they must be finely subdivided. Therefore a good meat grinder is essential in laboratories where much toxicological work is done. Small amounts of tissue may be cut finely with a knife or large scissors.

II. Classification of Poisons.—For toxicological purposes, poisons are best classified by the means used in separating them, rather than by their chemical nature.

Group 1. Compounds volatile with steam from aqueous media.

Group 2. Organic compounds not volatile with steam and best separated by extraction with solvents.

Group 3. Poisonous metals, detectable either directly or after admixed organic matter has been destroyed.

Group 4. Poisonous substances not falling in the previous three groups and requiring special methods for detection.

DETECTION OF POISONS

I. Poisons of Group 1, Volatile Poisons.—If the stomach contents or vomitus are used, test the reaction with litmus paper. Determine and record the odor. Look for any foreign material other than food, such as undissolved pills, colored salts such as copper arsenate, and the like. Observe and record the types of food present and degree of digestion. This information is sometimes of importance in determining the character of the last meal and the time since it was taken. Use half of the stomach contents, plus an equal volume of water for the distillation. The brain is the best organ to use, if volatile poisons are to be detected in solid tissue. Use 250 to 500 gm. finely divided with a meat chopper, suspending it in an equal weight of water.

densed distillate to the bottom of the receiver with the condenser outlet dipping into water, to prevent loss of the more volatile substances. Volatile acidic and neutral compounds distil readily from the weakly acidified mixture. If basic substances such as ammonia, coniine, or nicotine are suspected, the mixture should be made *alkaline* with sodium hydroxide before distilling.

B. Analysis of Distillate.—Record the volume of the distillate and its appearance. Substances immiscible with water may be present as an emulsion. Smell the distillate. Many substances volatile with steam have a characteristic odor.

1. **Alcohols and Aldehydes.**—(a) *Preliminary Test.*—Use 8 cc. of the distillate for the quantitative alcohol determination given on page 342. If there is no reduction of the dichromate reagent, the distillate is negative for methyl and ethyl alcohols, formaldehyde, acetaldehyde, and paraldehyde. If reduction occurs, apply the tests below to differentiate alcohols and aldehydes. If aldehydes and methyl alcohol are not detected, the results of the quantitative determination indicate the amount of ethyl alcohol present.

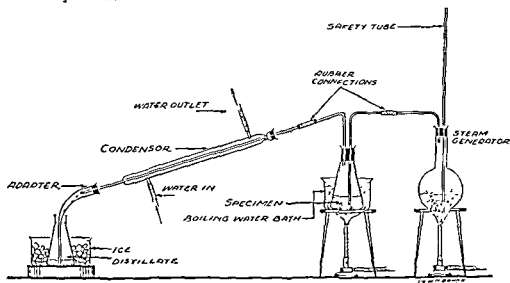


FIG. 29.—Steam distillation apparatus. *Note:* To facilitate addition and removal of solid matter, the specimen is best distilled in an Erlenmeyer flask. To prevent possible loss of very volatile compounds, the end of the adapter should be immersed in a thin layer of water at freezing temperature.

color at room temperature. A pink or violet color appearing within ten minutes indicates the presence of formaldehyde, the intensity of the color and the rapidity of its appearance being roughly proportional to the amount present. Both formaldehyde and acetaldehyde give this test.

(c) *Preparation of Schiff's Reagent.*—Add 0.5 gm. of basic fuchsin to 500 cc. of water and pass in sulfur dioxide until the weight has increased by 5 gm. Let the solution stand overnight to dissolve as much of the suspended fuchsin as possible. Make up to a volume of 1 liter. To remove brown discoloration, add 1 gm. of decolorizing carbon or activated charcoal, mix well, and filter by gravity. The filtrate should be water-white, if not, repeat the carbon treatment. The reagent will keep indefinitely in a glass-stoppered bottle. The amount of sulfur dioxide may be reduced to 1 gm., but this gives a reagent which is too sensitive for most purposes.

(d) *Denigès' Test.*—To 5 cc. of distillate add 1 cc. of concentrated sulfuric acid and cool to room temperature. Add 0.5 cc. of Schiff's reagent. A pink or violet color appearing within ten minutes indicates formaldehyde. Acetaldehyde does not react in the presence of sulfuric acid.

(e) *Rimini's Test*.—To 5 cc. of distillate, add 10 drops of freshly prepared 5 per cent phenylhydrazine hydrochloride solution, 1 or 2 drops of freshly prepared 0.5 per cent sodium nitroprusside solution, then 10 drops of 10 per cent sodium hydroxide. Formaldehyde will give a blue color, acetaldehyde a red color, a mixture of the two will give a blue to black color.

(f) *Note on Testing for Aldehydes and Alcohols*.—If formaldehyde was taken suicidally, a positive test for it may be obtained in stomach contents and solid tissues. However, formaldehyde in the tissues is usually due to its use as an embalming agent, as a contamination introduced at autopsy, or as a preservative or fixative for the organs. If paraldehyde was ingested, it appears in the distillate mainly in the form of acetaldehyde. It can also be detected by its characteristic odor. In contrast to ethyl alcohol (see page 342), methyl alcohol (wood alcohol) is not oxidized rapidly in the body, and if present in any appreciable amount, can be detected even twenty-four hours after ingestion. It may also be detected physiologically by its toxic effects, of which blindness is the most important.

(g) *Qualitative Test for Methyl and Ethyl Alcohols*.—If the aldehyde tests were negative, but methyl or ethyl alcohols are suspected, run the following test, which is based upon the oxidation of these alcohols to the corresponding aldehydes. Place 10 cc. of the distillate in a test tube, cooled in an ice-water bath. Bend a piece of copper wire around a pencil to obtain a spiral of 8 inches of straight wire to use as a test tube. Heat the spiral just to redness in the oxidizing flame of a burner six to eight times and plunge it into the 10 cc. of distillate each time. Reduction of the hot copper oxide on the wire produces acetaldehyde from ethyl alcohol and formaldehyde from methyl alcohol. Apply the tests for aldehydes; if positive, the corresponding alcohol is present in the distillate. Alcohols other than methyl and ethyl are rarely encountered. For their identification, more advanced texts must be consulted.

2. **Volatile Halogenated Compounds**.—Chloroform poisoning is encountered in some suicides and homicides. It is less used than formerly as an anesthetic. Chloral hydrate is still used medicinally, and is sometimes employed in "knockout drops." Carbon tetrachloride is occasionally encountered, bromoform and iodoform rarely.

(a) *Schwarz's Test*.—To 10 cc. of distillate, add 0.1 gm. of resorcinol and a few drops of 10 per cent sodium hydroxide. Heat on a water bath for ten minutes. A pink to red color is positive, and may indicate chloroform, or chloral hydrate. Iodoform also gives the test.

(b) *Fujiwara's Test*.—To 5 cc. of distillate, add 1 cc. of pyridine and 1 cc. of 10 per cent sodium hydroxide. Heat on a water bath for ten minutes. Chloroform or chloral hydrate give a pink to deep red color. Iodoform also gives the test.

(c) *Test for Chloroform*.—To 5 cc. of distillate, add 2 drops of aniline, and a piercing odor of chloroform hydrate.

(d) *Differentiation of Chloroform from Chloral Hydrate*.—To 5 cc. of distillate add 3 drops of a saturated resorcinol solution and 0.5 cc. of saturated sodium carbonate. After thirty minutes, add an equal volume of water. Chloral hydrate forms a green fluorescent solution while chloroform gives no fluorescence.

3. **Cyanides.**—If cyanide is suspected, divide the distillate into two portions, and apply Liebig's test and the Prussian blue test. Cyanides can frequently be identified by the characteristic odor of hydrocyanic acid.

(a) *Liebig's Test.*—To about 50 cc. of distillate, add 3 cc. of ammonium polysulfide test solution (U.S.P.) and 2 drops of 10 per cent sodium hydroxide solution. Evaporate to dryness on a steam bath, to form thiocyanate. Acidify with 5 cc. of 5 per cent hydrochloric acid, warm the solution and stir with a rod. Cool and filter. Refilter if necessary until the solution is clear. To the filtrate add 5 drops of 10 per cent ferric chloride solution. A red or deep orange color, due to ferric thiocyanate, is positive for cyanide, a yellow color is negative. The red color obtained in a positive test is soluble in ether.

(b) *Prussian Blue Test.*—To about 50 cc. of distillate, add 1 cc. of 10 per cent hydroxide solution. Concentrate on a steam bath to about 5 cc. Transfer to a test tube, washing the evaporating dish with 1 to 3 cc. of water. Add 5 drops of saturated ferrous sulfate solution and 1 drop of 10 per cent ferric chloride solution. Shake well, and heat gently. Add hydrochloric acid dropwise until the brown precipitate of ferric hydroxide just redissolves. If cyanide was present, a blue color will remain. If the amount was large, a blue colloidal solution or even a blue precipitate will appear. If the solution is green-blue, the amount of cyanide was small. Filter through a small filter paper. Any blue deposit on the paper serves to confirm cyanide.

in suicides,
in cases, the
Accidental

deaths sometimes occur from the use of HCN gas as a fumigant. Appreciable amounts of cyanide in the brain and other tissue indicate absorption during life. If taken by mouth, most of it will be found in the stomach; if inhaled, most of it will be found in the lungs.

4. **Phenolic Compounds.**—(a) *Millon's Reaction.*—All the commoner phenolic compounds give a positive Millon test. To 5 cc. of the distillate, add 1 cc. of Millon's reagent. If no color appears, heat just to boiling. A red color or a red precipitate indicates phenol or a phenolic compound. Cresol gives the test, and other less commonly encountered compounds which give similar colors are salicylic acid, methyl salicylate on warming, alpha- and betanaphthols, thymol, and eugenol. Almost all distillates from putrefied material give a positive Millon's test, owing to the formation of phenolic compounds in putrefaction. The test is also given by proteins owing to the presence of tyrosine, hence it may be misleading if applied directly to tissues, food, or body fluids. Millon's reagent is prepared by dissolving mercury in double its volume of nitric acid and adding one volume of water.

(b) *Ferric Chloride Test.*—To 5 cc. of distillate add a few drops of 10 per cent ferric chloride solution. Phenol (carbolic acid) gives a blue-violet color. The cresols give a green to blue color.

(c) *Bromine Water Test.*—To 5 cc. of distillate, add saturated bromine water until the solution has a permanent light yellow color. Phenol gives a heavy white precipitate of tribromophenol or tribromophenol hypobromite, depending upon conditions. Cresols also give brominated precipitates.

(d) *Discussion of Phenols.*—Carbolic acid and lysol are the phenolic substances most commonly encountered. The latter consists of a solution of cresols in soap. Taken internally, it gives the stomach contents a soapy appearance and a persistent foam on shaking. Phenols are quite frequently taken suicidally or accidentally, in which case the tissues of the mouth and gastro-intestinal tract usually show severe burns. The urine shows a characteristic dark "smoky" color.

5. Phosphorus.—(a) *Detection in Stomach Contents or Tissues.*—White phosphorus has a characteristic odor, which is readily detected in acute poisoning. In recent poisoning, it is usually more readily detected in the gastro-intestinal contents than in tissue. If white phosphorus is suspected, conduct the distillation for volatile poisons in a dark room. If a white vapor with a phosphorescent glow appears in the vapors in the flask or in the liquid in the condenser or receiver, phosphorus is present. In certain cases, the glow may be due to the non-toxic phosphorus sulfide found in ordinary strike-anywhere matches. For a confirmatory test, add a few drops of nitric acid to the distillate and evaporate to dryness. This oxidizes any phosphorus which may be present, to a residue of phosphoric acid. Dissolve in about 5 cc. of water, add a few drops of ammonium molybdate and warm gently. A yellow precipitate of ammonium phosphomolybdate is a positive test for phosphorus.

(b) *Discussion of Phosphorus.*—Phosphorus poisoning is now rarely encountered. Most cases arise from suicidal or accidental ingestion of white phosphorus pastes used in exterminating rats, or more rarely from the use of illicit phosphorus preparations of supposed aphrodisiac power. In cases which live for several days, phosphorus often cannot be detected chemically. The pathological findings are intense jaundice accompanied by acute yellow atrophy of the liver.

II. Poisons of Group 2, Non-Volatile Organic Substances.—A. *Extraction from Tissues and Body Fluids.*—The residue of stomach contents or organs left in the flask after the distillation of volatile poisons, may be used for the isolation of non-volatile organic poisons, since the hot water dissolves out water-soluble materials, while water-soluble proteins are coagulated by the heating. While still hot, strain the suspension through four layers of gauze, squeezing out as much of the liquid from the solid material as possible. Filter the turbid liquid through moist absorbent cotton or filter paper. The clear filtrate should be acid, if not, add tartaric acid. If filtration is slow, pour all the liquid into 2 to 3 volumes of ethyl alcohol, and filter after standing for fifteen minutes. This will precipitate much protein, and aid filtration.

Evaporate the filtrate on a steam bath to a sirup about 50 cc. in volume. Slowly add 5 volumes, about 250 cc., of 95 per cent ethyl alcohol, stirring well with a rubber policeman on a glass rod, to keep the white precipitate (mainly protein) in a finely divided state. After standing for an hour, filter through cotton or filter paper moistened with alcohol. Evaporate the alcoholic filtrate to a sirup. Add about 50 to 75 cc. of distilled water, rub the residue with a policeman on a glass rod, warming for about ten minutes on a steam bath. After about thirty minutes, filter through filter paper, rinse the dish, then the filter with 25 cc. of water. The clear combined filtrate should be acid.

Some alkaloids such as aconitine, physostigmine, apomorphine, cocaine

and some glucosides, are destroyed or chemically altered by heat and acidity. If such poisons are suspected, make a direct alcoholic extract of the ground organs or stomach contents with 4 to 5 volumes of 95 per cent alcohol acidified with tartaric acid. Let the mixture of tissue and alcohol stand overnight, then pour off most of the alcohol and wash the semi-solid residue with one volume of alcohol. Squeeze out the solid material in gauze, filter through paper, then evaporate to a sirup under reduced pressure or by gentle warming in an air current from a fan. Dissolve in water and filter as in the regular procedure. All non-volatile organic substances can be isolated by a direct alcohol extraction.

B. Isolation and Identification.—Routinely, the 100 cc. of aqueous solution may now be extracted three times: (1) From acid solution with ether to obtain acidic and neutral substances; (2) from basic solution with ether to obtain basic substances; and (3) from ammoniacal solution with chloroform-alcohol mixture, or with ethyl acetate, to obtain morphine and narceine. In some cases it will not be necessary to make all three extractions, but only those indicated by circumstances.

1. Extraction of Acidic and Neutral Compounds.—The 100 cc. or more of weakly acid aqueous solution, is extracted twice with an equal volume of ether in a separatory funnel, shaking each time for about a minute. The ether extracts are combined in a separatory funnel, and any drops of water allowed to settle out. The combined extract is then shaken for one minute with 30 cc. of approximately 0.1 N hydrochloric acid to remove traces of water containing ether-insoluble substances, the acid washings being discarded. The original acidic aqueous solution should be retained for the extraction of basic compounds and morphine, if necessary.

Evaporate successive portions of the ether extract on a steam bath in a small beaker. The residue may contain neutral or acidic substances such as barbiturates, salicylates, acetanilide, acetophenetidine, antipyrine, salol, picric acid, dinitrophenol, pyramidon (aminopyrine), caffeine, picrotoxin, and the like.

2. Tests for Neutral and Acidic Compounds.—The amount of solid residue obtained will vary considerably, depending on the poison taken, the amount, and the time elapsed before death. In routine analyses, all the compounds below may be tested for, or individual compounds may be tested for as indicated.

(a) *Barbiturates.*—To all or part of the residue, which must be completely dry, add 2 cc. of chloroform. Run 0.5 cc. into a small diameter tube such as a 1-cc. centrifuge tube or a 10 x 75 mm. fermentation tube. Add 1 drop of 1 per cent solution of cobalt acetate in absolute methanol. Mix well. Then using a medicine dropper or pipet, run about 0.5 cc. of 5 per cent isopropylamine in absolute methanol carefully down the side of the tube to form a layer on the chloroform solution. A purple color at the interface of the liquid layers, more easily seen with a white sheet of paper held behind the tube, is a positive test for barbiturates. A yellow or green color is negative.

The test may likewise be applied to the chloroform extract of a residue obtained by ether extraction of urine. If cobalt acetate is not available,

The barbiturate drugs can be roughly divided into two groups, those with a prolonged hypnotic action, and those with a short action. The former are more readily detected chemically in urine and in tissues, the latter are less so, their short action being due to the fact that they are rather rapidly and completely broken down in the body. It is frequently desirable to identify the specific barbiturate found in a given case. This is most readily done by subliming the extraction residue in a vacuum and determining the melting point of the sublimate. Details of these advanced methods are beyond the scope of this book.

(b) *Salicylates, Pyramidon, Antipyrine*.—Evaporate the remaining chloroform solution to dryness on a water bath, then add 5 cc. of water and heat about a minute longer. To 1 cc. of this solution, add 1 to 3 drops of 10 per cent ferric chloride solution. Salicylates or pyramidon give a blue-violet color, antipyrine gives a red color. If positive, test 1 cc. with Millon's reagent. Salicylates give a pink to red color on warming. If negative, add 2 to 3 drops of dilute silver nitrate solution. A black, blue, or violet color due to colloidal silver indicates pyramidon (aminopyrine). Nitric acid will discharge the color.

(c) *Acetanilide and Acetophenetidine*.—To 2 cc. of a water solution of residue, add 2 cc. of hydrochloric acid. Boil for about five minutes, to hydrolyze. Cool, and add 1 cc. of a cold, saturated solution of phenol, then add drop by drop a freshly prepared, filtered solution of calcium hypochlorite. Either of the compounds gives a pink to red color of indophenol, which becomes blue or violet when concentrated ammonia is added.

To 1 cc. of a water solution, add 1 cc. of a saturated alcoholic potassium hydroxide solution. Heat gently for about a minute. Add 1 drop of chloroform. Acetanilide, but not acetophenetidine, gives a piercing odor of phenyl isocyanate.

(d) *Picric Acid and Dinitrophenol*.—These give a yellow color in aqueous solution, which increases in intensity on adding alkali. The aqueous solution of either compound will dye wool or silk but not cotton.

form of an oil. The water
h ferric chloride, while its

ic Compounds.—To permit a more positive identification of any of these compounds, the residue, if enough is obtained, may be purified by sublimation under reduced pressure, or by recrystallization from water or other solvents. After purification, the melting point is determined, the crystalline form studied, or derivatives prepared.

3. *Extraction of Alkaloids and Other Basic Substances*.—The acid aqueous solution after the extraction of acid and neutral substances, is now made alkaline by adding sodium hydroxide solution. The alkali liberates alkaloids and other basic compounds from their salts, and combines with phenolic compounds such as morphine. Extract the alkaline liquid with ether as before, but wash the combined ether extract with 30 cc. of 0.1 N sodium hydroxide, discarding the washings. Evaporate successive portions of the ether extract in a 100-cc. beaker. The residue may contain such basic substances as: Quinine, codeine, heroin, strychnine, atropine, cocaine,

nicotine, brucine as well as less common alkaloids. Some caffeine, anti-pyrine, and pyramidon may separate in this extraction as well as in the previous extraction of the acid aqueous solution.

4. **Extraction of Apomorphine.**—Apomorphine is so rarely encountered that in routine analyses it is not necessary to run an extraction for it. If its presence is suspected, make the alkaline aqueous solution neutral or slightly acid with hydrochloric acid, then weakly alkaline with a little ammonia. Extract with ether in the usual fashion. Otherwise extract immediately for morphine.

5. **Extraction of Morphine.**—Extract the ammoniacal aqueous solution four times with warm chloroform containing about 10 per cent by volume of ethyl alcohol, or better, with ethyl acetate. Shake the combined extracts in a separatory funnel with a little anhydrous sodium sulfate or sodium chloride to remove water, then evaporate successive portions of the chloroform in a small container. The residue contains any morphine or narceine.

6. **Tests for Alkaloids.**—Preliminary tests for alkaloids are of two sorts, precipitation reactions and color reactions. Although a large literature on the subject exists, most of the tests, particularly the color reactions, are of limited value. In putrefied material various amines, ptomaines or pseudo-alkaloids may be found. They may give some but not all of the reactions of a particular alkaloid. Therefore, a considerable number of tests must be applied before any alkaloid can be confirmed conclusively. Some specific property, such as the melting point, should be determined if at all possible. Very small amounts of certain alkaloids may be fatal, so that it is frequently difficult or impossible to isolate appreciable amounts from dead bodies.

The color tests for alkaloids are mainly based upon a rather complete disintegration of the alkaloid molecule by strong oxidizing agents such as nitric or sulfuric acids. Frequently some organic compound is present to react with the fragmentation products to give a color. Unfortunately, the course of the reaction in the color tests is profoundly influenced by the ratio of alkaloid to reagent, state of subdivision of the alkaloids, temperature, presence or absence of moisture, and the presence or absence of extraneous substances giving false colors. Small amounts of alkaloids are often very difficult to isolate, so that application of color tests to partly purified material gives only brown smears. The precipitation reactions are generally of greater value than the color tests. Table 48 gives in tabular form the reactions of the more important alkaloids and glucosides.

7. **Precipitation Reagents.**—Some of the more useful of these reagents are:

- (a) *Picric Acid*, saturated aqueous solution.
- (b) *Picrolonic Acid*, a 0.1 N solution in alcohol.
- (c) *Gold Chloride*, 5 per cent aqueous solution.
- (d) *Platinic Chloride*, 5 per cent aqueous solution.
- (e) *Wagner's Reagent*.—Dissolve 10 gm. of potassium iodide and 1 gm. of iodine in 100 cc. of water.
- (f) *Marme's Reagent*.—Dissolve 20 gm. of potassium iodide in 20 cc. of boiling water, add 10 gm. of cadmium iodide, and dilute to 100 cc.
- (g) *Mayer's Reagent*.—Dissolve 1.35 gm. of mercuric chloride and 5 gm. of potassium iodide in 100 cc. of water.

TABLE 48—REACTIONS OF THE MORE IMPORTANT ALKALOIDS AND GLUCOSIDES
(Reprinted from Merck's Index, 4th ed., courtesy of Merck & Co., Inc.)

ABBREVIATIONS

Bl = blue
bl = bluish
Bk = black
bk = blackish
Br = brown
br = brownish
Ca = carmine
ch = cherry
Cr = crimson
dk = dark
dt = dirty
exs = excess

G = green
g = greenish
In = indigo
lm = lemon

lt = light
ol = olive
Or = orange
ppt = precipitate
Sc = scarlet

P = purple
R = red
r = reddish
Sc = scarlet

soln = solution
V = violet
Y = yellow
y = yellowish

Wh = white
wh = whitish
= to
Δ = changing to

	COLOR REACTIONS						PRECIPITATES				VARIETY OTHER REACTIONS
	Conc. H_2SO_4	Conc. HNO_3	$H_2SO_4 + H_2O$ (Estermann)	$H_2SO_4 + Ammonia$ (for test 100-1-3 (trichloride))	$H_2SO_4 + Ammonia$ (Vanadate 200-1-3 (Mandelin))	$H_2SO_4 + Potassium persulfate 200-1-3 (Merck)$	$H_2SO_4 + Formaldehyde 200-1-3 (Marquet)$	$H_2SO_4 + H_2O$	$H_2SO_4 + H_2O$	$H_2SO_4 + H_2O$	
Aconitine	color purp. + unquenchable by heat	color—Br	color—Y + Br	color—Y + Br	lt Br—Or	color—Y + Br	color	Wh. lt soln	Y (1:40,000)	Br	ppt only in conc. soln
Apomorphine	color—Br—R	P—Or	R—Or	GAY	GABl	VdBr by heat	color	Y only ppt Δ	1:10,000	blood fl.	Y
Atropine ...	color	color	color	color	RdY	color	color	1:7000	1:10,000	color	Y only in conc. soln (1:200)
Berberine	color by heat	lt—R	lt—R	VdBr—Δ G	Δ GABl	color	color—Br	Wh. amorph.	Or R	Br	Y amorph. (1:2000)
Berberine ...	ol. GAY, Δ G by heat	ol. Δ G	ol. Δ G	br. GAY, Δ G by heat	Δ G Br	g—Δ G—R	lt R	Y G	Or R (1:5000)	R	lt Y (1:5000)
Brucine	color Δ Y by heat	color	color	RdY, Δ color	Y Or	Y RdY by heat	lt R	Wh. Y (1:5000)	Δ (1:5000)	Br (1:50,000)	Cryt (1:10,000)
Caffeine ...	color	color	color	color	color	color	color	none	none?	color	none or only in conc. soln
Cinchonidine	color	color	color	color	color	color	color	color	color	color	color
Cinchonine ...	color	color	color	color	color	color	color	color	color	color	color
Cocaine ...	color purp. + unquenchable by heat	color	color	color	Or	color	color	1:140,000	Br (1:50,000)	Br	Y (1:1000)

	COLOR REACTIONS						PRECIPITATES				VARIOUS OTHER REACTIONS
	Conc. H_2SO_4	Conc. HNO_3	$H_2SO_4 + H_2O_2$ (Erdmann)	$H_2SO_4 + (NH_4)_2S$ (Froehde)	$H_2SO_4 + (NH_4)_2CO_3$ (Marignolle)	$H_2SO_4 + H_2O_2$ (Marignolle)	$H_2SO_4 + H_2O_2$ (Marignolle)	$H_2SO_4 + H_2O_2$ (Marignolle)	$H_2SO_4 + H_2O_2$ (Marignolle)	$H_2SO_4 + H_2O_2$ (Marignolle)	
Cocaine ..	colorless by heat by HNO_3	Y-R	colorless by heat	colorless by heat	colorless by heat	colorless by heat	colorless by heat	colorless by heat	colorless by heat	colorless by heat	$H_2SO_4 + FeCl_3 =$ deep bl $H_2SO_4 + CuCl_2 =$ R $H_2SO_4 + K_2CrO_7 =$ Cd G
Colechicine	Y-R by heat	Y-R by heat	Y-R by heat	Y-R by heat	Y-R by heat	Y-R by heat	Y-R by heat	Y-R by heat	Y-R by heat	Y-R by heat	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Coniine .	color	Y-color	color	color	color	color	color	color	color	color	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Curarine	bl-R	P	Y-R	Y	Y	Y	Y	Y	Y	Y	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Diacetylmorphine	color	Y-R by heat	Y-R by heat	Y	Y	Y	Y	Y	Y	Y	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Digitalin .	bl-R by heat	Y-R by heat	Y-R by heat	Y	Y	Y	Y	Y	Y	Y	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Emetine	colorless by heat by KOH	Or Y	Y	Y	Y	Y	Y	Y	Y	Y	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Ethylmorphine ..	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Gelsemium	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Hydrastine .	colorless by heat by KOH	Or	Y-R	Y	Y	Y	Y	Y	Y	Y	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Hyoscyamine (Scopolamine)	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Hyoscyamine	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Labelline .	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Morphine .	colorless by heat by KOH	Or R	Y-R	Y	Y	Y	Y	Y	Y	Y	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Narcotine ..	colorless by heat by KOH	Y-R	Y-R	Y	Y	Y	Y	Y	Y	Y	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Narcotine	colorless by heat by KOH	Y-R	Y-R	Y	Y	Y	Y	Y	Y	Y	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G
Nicotine .	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	colorless by heat by KOH	$H_2SO_4 + K_2CrO_7 =$ Cd G $H_2SO_4 + K_2CrO_7 =$ Cd G

TABLE 48 — REACTIONS OF THE MORE IMPORTANT ALKALOIDS AND GLUCOSIDES—Continued

	COLOR REACTIONS							PRECIPITATES				Various Other Reactions
	Conc. H_2SO_4	Conc. $LiNO_3$	H_2SO_4 + HNO_3 (Erdmann)	H_2SO_4 + Ammon. Vanadate 20:1 (Grobale)	H_2SO_4 + Ammon. Vanadate 20:1 (Mandel)	H_2SO_4 + Selenium Acid 200:1 (Meyer)	H_2SO_4 + Formaldehyde (Marques)	H_2O : 2KI	$BaCl_2$: 4KI	1 KI	Potash Acid	
Papaverine	color—pale P dark by heat	y—dk R	gdkdk R	Gdkdk Vdk R by heat	ld G—Bl	gdkdk Bl V by heat	Rdk	Wk (1:1000)	Or R (1:1000)	dk R (1:50,000)	Y (1:500)	Clearer = g dk Br by NH_4OH
Physostigmine	color—Y	Ydk Gdk R	Ydk R	color	Ydk Gdk R by heat	color by heat	color—Ydk R	Wk (1:10000)	ppt	p Br (1:22,000)	only in conc soln	Alkali hydroxides = intense R Ydkdk
Pilocarpine	color		color	color	Ydk Gdk R	color	color—Ydk R	Ydk	ppt	ppt	ppt	H_2SO_4 + K_2CrO_7 = G color
Piperine	blood fluid dk	Oxidized R by heat	gdkdk gdk by heat	Rdkdk Rdkdk	blood fluid	gdk	R	Ydk	ppt	ppt	ppt	
Quinidine	color	color	color	color	ld G	color	dk G	1:5000	Weak Rdkdk Wk	Br	ppt	Clearer + NH_4OH = no under Quinine H_2SO_4 + K_2CrO_7 = Y Wk ppt
Quinine ...	color—lt ydk Br by heat	color	color	color	color	color by heat	color	Wk (1:125,000)	Or R (1:50,000)	p Br	Amorph (1:100,000)	Clearer + NH_4OH = G dkdk sol in conc (neutral Gdkdk), soln in conc (acid Gdkdk), soln NH_4OH = dk—Ydk Y K_2CrO_7 = Y ppt Peroxide acid liberates free I
Solanine...	Brdk V	color	Ydkdk R	Ordkdk Y	Ordkdk V	Ydkdkdk Br by heat	Br	none	Y	none in acid soln	ppt in conc soln	
Sparteine	color	color	color	color	color	color	none	none	none	none	none	
Strophanthin	R: Ydkdkdk	R: dkdk series of colors by heat						none	none	none	none	H_2SO_4 + K_2CrO_7 = gdk HCl = Ydk series of colors by heat HCl = lt Br K_2CrO_7 = g Y color or all ppt
Strychnine	color—Or Y by KOH	color	color	color	Vdkdk R	color	color—dkdk by heat	dk (1:15,000)	lt Y (1:250,000)	dk R (1:50,000)	Y (1:20,000)	H_2SO_4 + K_2CrO_7 = Vdkdkdk HCl = dkdk R
Theobromine	color	color	color	color	color	color	color	none		ppt in conc soln	none	Evapor. Y Clearer or HNO_3 & soln NH_4OH = Y color (neutral test)
Thebaine	blood fluid R	Y	R	Or				ppt (1:5000)	Or (1:10,000)	ppt (1:6000)	Y	
Veratrine...	Ydkdkdk by heat	Y	Y—Ordkdk R	Ydkdkdk R	Ydkdkdk R	in Ydkdk Gdk by heat	Ydkdkdkdk by heat	p Wk in conc. soln.	lt dkdkdk Y in conc soln	Br (1:15,000)	1:10,000	H_2SO_4 + K_2CrO_7 = dkdk H_2SO_4 + sugar = gdkdk deep Gdk HCl = deep Ydkdkdk R by heat
Yohimbine ..	color—lt G by K_2CrO_7	color—Aukdk Ydkdk R by heat	ld Br dk— Ydk	Bl	Bl	lddk	dk Gdkdk Y by heat	Wk			Y	

TABLE 48.—REACTIONS OF THE MORE IMPORTANT ALKALOIDS AND GLUCOSIDES—Continued

Supplementary List of Tests of Alkaloids & Glucosides

Adonidin.....	$\text{H}_2\text{SO}_4 = \text{deep R.} - \text{HNO}_3 = \text{In Bl.} - \text{HCl} = \text{lt R.} - \text{Ppts w. AuCl}_3 \text{ HCl \& picric acid}$
Arecoline	$\text{HgI}_2 \text{ 2KI} = \text{Y.} - \text{BiI}_3 \text{ 4KI} = \text{R} - \text{AuCl}_3 \text{ HCl} = \text{Y only ppt} - \text{Picric acid} = \text{tarry ppt.} - \text{Tannin ppts alkaloid but not the salts}$
Aspidospermine	Mandelin = lt P Δ 3 R Δ PR — Hot HCl = R. — $\text{K}_2\text{Cr}_2\text{O}_7 = \text{rBr } \Delta \text{ dk G.}$ — $\text{HgI}_2 \text{ 2KI} = \text{Y flocks.} - \text{Picric acid} = \text{Y ppt.}$
Coltarine	$\text{HNO}_3 = \text{R.} - \text{H}_2\text{PtCl}_6 = \text{Y ppt} - \text{Ferrous salts \& CuSO}_4 \text{ give ppts}$
Digitoxin	Froehde = Br. — Mandelin = VBr — Mecke = VBr. — Marquis = Br.
Ephedrine	Millon ($\text{Hg}[\text{NO}_3]_2$), $\text{AuCl}_3 \text{ HCl}$, H_2PtCl_6 , & $\text{BiI}_3 \text{ 4KI}$, afford charact. <i>cryst. ppts</i>
Ergotamine	$\text{H}_2\text{SO}_4 = \text{Y } \Delta \text{ VBl.} - \text{Gives ppts w. 1 KI, HgI}_2 \text{ 2KI, picric acid, \& tannin}$
Homatropine	Evap'g then add'g <i>alcoh.</i> KOH = Y. — $\text{HgI}_2 \text{ 2KI} = \text{wh ppt.} - \text{Picric acid} = \text{Y ppt.} - \text{Alcoh. solut. HgCl}_2 \text{ w. free base} = \text{R.}$
Pelletierine.....	$\text{H}_2\text{SO}_4 + \text{K}_2\text{Cr}_2\text{O}_7 = \text{G.} - \text{AuCl}_3 \text{ HCl} + \text{heat} = \text{Au ppt (reduction)} - \text{Tannin} = \text{ppt sol. in excess}$
Picrotoxin.....	$\text{H}_2\text{SO}_4 = \text{Y.} - \text{H}_2\text{SO}_4 + \text{K}_2\text{Cr}_2\text{O}_7 = \text{V.} - \text{H}_2\text{SO}_4 + \text{KNO}_3 + \text{excess Na}_2\text{CO}_3 = \text{vermillion R.} - \text{Reduces Fehling's soln} - \text{Froehde} = \text{Or.} - \text{Mandelin} = \text{gY.} - \text{Marquis} = \text{lt ch R.}$
Salicin	$\text{H}_2\text{SO}_4 = \text{R. \& R ppt forms on add'g water} - \text{Froehde} = \text{V } \Delta \text{ dk ch.} - \text{H}_2\text{SO}_4 + \text{K}_2\text{Cr}_2\text{O}_7 + \text{heat} = \text{odor of salicylaldehyde.}$
Theophylline.....	$\text{H}_2\text{SO}_4 = \text{Y } \Delta \text{ Or } \Delta \text{ blood R.} - \text{Evap'd w. Cl-water} = \text{bright R } \Delta \text{ V by NH}_4\text{OH.}$

(h) *Dragendorff's Reagent*.—Dissolve 8 gm. of bismuth subnitrate in 20 cc. of concentrated nitric acid. Pour this into a saturated solution of 22.7 gm. potassium iodide. Let stand about three hours for potassium nitrate to crystallize out, then decant the solution and dilute it to 100 cc.

8. *Color Reagents*.—Some of the more important color reagents are:

(a) *Sulfuric Acid*, concentrated.

(b) *Nitric Acid*, concentrated.

(c) *Erdmann's Reagent*.—To 20 cc. of concentrated sulfuric acid add 10 drops of a solution of 10 drops of concentrated nitric acid in 100 cc. of water.

(d) *Froehde's Reagent*.—Dissolve 5 mg. of molybdic acid or sodium molybdate with gentle warming in 1 cc. of concentrated sulfuric acid. The solution should be colorless. It does not keep long.

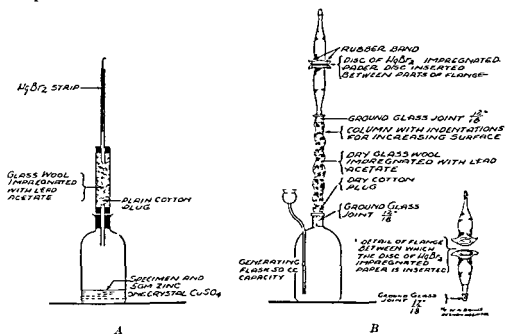
(e) *Mandelin's Reagent*.—Dissolve 1 gm. of finely powdered ammonium vanadate in 200 gm. of concentrated sulfuric acid.

(f) *Marquis' Reagent*.—Mix 2 to 3 drops of formaldehyde (regular 10 per cent solution) with 3 cc. of concentrated sulfuric acid. This reagent is unstable; prepare it as needed.

9. *Analysis of Alkaloidal Residues*.—The residue from the extraction of the alkaline solution with ether should be examined first for any characteristic odor of nicotine, coniine, pyridine, or aniline. All are liquids which may appear at this point in the analysis. Take up the residue in 4 cc. of ether and divide into two portions.

(a) *Negative Control*.—Add 0.1 gm. of arsenic-free sodium bisulfite to 20 cc. of 10 per cent by volume sulfuric acid or a quantity equivalent to that used in the digestion, then boil, cool, add stannous chloride, etc., as at the close of the digestion.

(b) *Positive Control*.—Made in the same way as the negative control but with the addition of a known amount of standard arsenic solution before adding the sodium bisulfite. The arsenic stock solution contains 1 mg. of As per cc. Dissolve 1.319 gm. of As_2O_3 in 15 cc. of 1 N NaOH. Dilute to 500 cc. Add 15 cc. of 1 N HCl and make to 1 liter. This solution is diluted down to standard solution as desired, usually to 1 microgram of As per cc.



Gutzeit appa-
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(c) *Unknown*.—Use 20 cc. of the digestion mixture or some aliquot of it.

or developed in the mercuric bromide strips. A yellow color merging to dark brown at the lower end, indicates the presence of arsenic.

By using different dilutions of standard arsenic solution and different dilutions of the unknown, matching the colors representing equal concentrations of arsenic and figuring back, it is possible to determine the approximate amount of arsenic in the unknown.

An improved form of the Gutzeit apparatus is shown in Figure 30. The essential improvement is the addition of a flange arrangement, which permits the use of a disk of paper, impregnated with mercuric bromide to be inserted between the flanges, so that the color resulting from the generation of arsine is deposited in the form of a spot instead of in a streak.

The sensitivity of the test is increased and a quantitative estimation of the arsenic may be made with greater accuracy. As little as 1 microgram of arsenic can be detected, and graduations of 1 microgram in arsenic concentration can be measured.

To prepare the test disks, immerse 180 mm. filter papers, Whatman's No. 1 or No. 4, for about five minutes in a 5 per cent solution of mercuric bromide in absolute methanol in a large Petri dish. Suspend the paper in the air to dry. Then cut off a $\frac{1}{2}$ -inch strip around the edge, and use the center portion for the disks.

6. Marsh-Berzelius Test.—The Marsh-Berzelius test permits the isolation of free arsenic, so that further tests can be applied to it. The arsine generated is decomposed to free the arsenic instead of being allowed to react with a mercuric salt. Place 30 gm. of arsenic-free zinc in the generator of the apparatus, shown in Figure 31. Pour 10 cc. of 10 per cent by volume sulfuric acid through the thistle tube. The evolution of hydrogen should not be too rapid. Cool the generator if necessary with a water bath.

At the same time, a shining ignition.

addition of unknown, to check the reagents for freedom from arsenic.

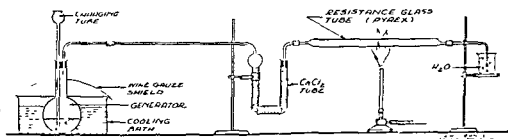


Fig. 31.—Marsh apparatus

Antimony forms a mirror in the Marsh test. The arsenic mirror is soluble in sodium or calcium hypochlorite solution, the antimony mirror is not. If the mirror is dissolved in concentrated nitric acid, and ammonium molybdate solution is added, arsenic gives a precipitate of ammonium arsenomolybdate, but antimony does not. If silver nitrate is added to the nitric acid solution and ammonia layered over it, arsenic will give a yellow precipitate of silver arsenate, antimony gives no such precipitate.

7. Antimony.—As a poison, antimony is similar to arsenic, but is more rarely encountered. In the Reinsch test, it forms a gray-black deposit on the copper spiral, very similar to that of arsenic, but upon subliming from the copper, it appears as an amorphous deposit instead of forming octahedral crystals of arsenic trioxide. In the Gutzeit test, it gives stibine, which produces a black color on the impregnated paper, instead of yellow or brown as with arsenic. Its behavior in the Marsh test has been described above.

8. Bismuth.—Poisoning with bismuth is rare. It is mainly of importance as an interfering substance in the Reinsch test, since its compounds are

used medicinally. The black deposit which it gives in the Reinsch test is difficult or impossible to sublime. If stomach contents or tissue are digested, as in the test for arsenic, bismuth will give an orange-yellow color or precipitate if some of the digest is diluted and placed on a filter paper impregnated with a solution of 1 gm. of cinchonine and 2 gm. of potassium iodide in 100 cc. of water containing a little nitric acid. It will give a yellow color in a dilute sulfuric acid solution containing a little potassium iodide and sodium sulfite.

9. **Lead.**—Acute lead poisoning is relatively rare, chronic lead poisoning is fairly common, arising from prolonged exposure to the metal in one form or another. For methods of collecting blood and urine for the determination of lead, see under "Special Methods."

IV. **Poisons of Group 4, Miscellaneous Poisons.**—1. **Oxalates.**—Poisoning may occur by ingestion of either oxalic acid or its salts. Large doses are rapidly fatal. Accordingly, oxalate can usually be recovered from the stomach contents. In the internal organs, the highest concentration is found in the liver. Some oxalic acid may be isolated by ether extraction of acidified aqueous solutions and will appear in Group 2. However, if there is reason for suspecting that oxalate has been taken, proceed as follows: Mix the finely divided material with 3 to 4 volumes of alcohol and acidify with hydrochloric acid. Let the mixture stand one to two hours with frequent stirring. Filter, washing the residue with alcohol. Add about 10 cc. of water to the filtrate and evaporate on a steam bath. Make the solution alkaline with ammonia and filter if necessary. Acidify with acetic acid. Add calcium chloride solution. If a precipitate appears immediately, oxalic acid is probably present. Calcium oxalate crystallizes in octahedrons having an "envelope" shape. It may be necessary to redissolve the precipitate in hot hydrochloric acid, filter and repeat the treatment with ammonia, acetic acid, and calcium chloride.

Oxalic acid may be confirmed by heating the calcium oxalate for a few minutes with dilute sulfuric acid. Add a 1 per cent solution of potassium permanganate in *small* drops from a capillary pipet. Oxalic acid will discharge the purple color of the permanganate solution.

2. **Mineral Acids.**—Nitric, sulfuric, and hydrochloric acids taken in strong concentrations cause corrosions and discolorations of the face, acid the parts are yellowish ring. If any doubt exists, test with Congo red paper which turns blue with even very dilute solutions of these acids, then proceed with the ordinary qualitative tests for these acids.

3. **Borates.**—Boric acid and its salts are moderately poisonous. In acute poisoning, gastro-intestinal irritation occurs, but no particularly characteristic signs appear at postmortem. Evaporate urine or finely ground tissue to dryness with sodium carbonate and potassium nitrate. Ignite at the lowest temperature which will produce complete combustion of carbonaceous matter. Acidify the ash with hydrochloric acid and dip a piece of turmeric paper in it. If boric acid is present the paper will have a red-brown color when dried, which changes to a greenish-blue when a drop of dilute sodium hydroxide is applied. For a flame test, some of the ash may be suspended in methanol or ethanol and a little concentrated sul-

furic acid added. In the presence of boric acid, the flame produced when the alcohol is ignited, is colored green.

4. **Chlorates.**—Potassium chlorate is used in certain toothpastes and gargles. A conspicuous feature of chlorate poisoning is the production of a chocolate-brown color in the blood, owing to the formation of methemoglobin. It is customary to extract chlorates from suspected material by dialysis. The finely-ground material is placed in a cellophane or other sack and dialyzed in a beaker of distilled water for five to six hours. The dialysate is evaporated to dryness on a steam bath, dissolved in a little water and filtered if necessary. To the solution add a drop of freshly distilled aniline and a little hydrochloric acid. If chlorate is present, a blue or green color will appear if the mixture is warmed gently.

5. **Nitrites.**—These may be encountered either as sodium nitrite or as brown fumes of nitric oxide. The nitrites also produce a brown discoloration of the blood by forming methemoglobin. The suspected material such as stomach contents is acidified and distilled. The distillate may be used to diazotize some aromatic amine, which is then combined with a coupling agent to give a red azo dyestuff.

6. **Iodine.**—Tincture of iodine is a popular agent in suicidal attempts. It can often be detected by the brown stains produced on the face or clothing. If any appreciable amount has been swallowed, it can usually be detected by filtering the liquid and shaking with

7. **Bromine.**—Acute poisoning with free bromine or alkali bromides is rare. Chronic bromide poisoning may result in rashes on the face and neck or in mental disturbances. For detection of bromides in blood, see under "Special Tests."

8. **Chlorine.**—Poisoning by ingestion of chloride of lime and similar preparations occasionally occurs. If some of the ingested material can be recovered before it has had time to react with organic matter, the smell of free chlorine may be observed. The production of a yellow color with α -tolidine by methods similar to those used in water analysis is another test.

9. **Fluorides.**—Owing to the prevalent use of fluorides as insecticides, acute fluoride poisoning is very common. Cramps, nausea, and vomiting shortly after eating the poisoned food, are the symptoms. The best test is that of Gettler. Place 1 cc. of stomach contents or 1 to 3 cc. of urine in a small porcelain crucible with sodium hydroxide solution and heat on a hot plate until dry. Mix a little powdered glass with the thoroughly dried material. Make the mixture acid with a little concentrated sulfuric acid, and cover the crucible immediately with a microscope slide or other sheet of glass having a drop of water on its lower surface. Heat the crucible gently (a heating block at 150° C. is best), placing a small beaker of ice water on the slide to keep it cool. After three to five minutes, remove the slide, and add a drop of 5 per cent sodium chloride to the drop. Crystals of sodium fluosilicate develop immediately or upon standing. They are hexagonal, and in a somewhat subdued light they have a faint pink color.

10. **Carbon Monoxide.**—The following method is of value as a quick, qualitative test for carbon monoxide in suspected acute poisoning. It is

of no value in suspected chronic poisoning. Quantitative methods for CO in blood and air are given in Chapter XVI.

To two 15-cc. portions of water in test tubes add 3 drops of suspected blood and 3 drops of normal blood. Mix well by inverting the tubes. If one tube has a much stronger color than the other, dilute it until the intensity, not the shade, of the colors is approximately equal. If large amounts of CO are present, the suspected blood will have a cherry-red color as compared to the deep-red color of the normal blood. With small amounts, the difference in color is not distinct.

To each solution add 5 drops of 10 per cent sodium hydroxide and mix well by inverting. Normal blood turns a greenish-brown color immediately, owing to the formation of alkali hematin. Blood containing CO changes color very slowly. With less than 20 per cent saturation with CO, the color changes in a few minutes, with 50 per cent saturation or above, the color will remain red for hours.

Considerable experience is required to detect less than 20 per cent saturation with CO. The blood of horses, or of humans less than six months old, is not affected by the addition of alkali.

SPECIAL METHODS

I. Ethyl Alcohol in Blood, Urine, or Spinal Fluid.—A modified Nicloux method is used. Ethyl alcohol reduces dichromate in acid solution to a green chromium salt. The amount of green color produced is a measure of the amount of alcohol. The dichromate reagent has been found to give better results if made up by a slightly different method than that given in the literature. For that reason it is called "Anstie's reagent, modified, stronger" and should not be confused with that described in other methods.

1. Reagents.—(a) *Anstie's Reagent, Modified, Stronger.*—Dissolve 3.70 gm. of pure potassium dichromate in 150 cc. of distilled water. Add slowly, with constant stirring, 280 cc. of pure concentrated sulfuric acid. Dilute to 500 cc. with distilled water.

(b) *Standard Alcohol Solution (2 per cent).*—Place about 50 cc. of distilled water in a 100-cc. volumetric flask. Add 2.53 cc. absolute ethyl alcohol with an accurately graduated pipet, holding the pipet tip near the surface of the water to prevent loss of alcohol by evaporation. Make up to 100 cc. with distilled water.

(c) *Scott-Wilson Reagent.*—Dissolve 90 gm. of sodium hydroxide in 300 cc. of water. When cool, add it to a solution of 5 gm. of mercuric cyanide in 300 cc. of water. Mix thoroughly, then add with constant stirring, a solution of 1.45 gm. of silver nitrate in 200 cc. of water. The reagent will keep for six months. If a turbidity or precipitate forms, filter it. **CAUTION.**—*Never pipet this reagent. It is very poisonous.* Use a 10-cc. cylinder for measuring it.

(d) *Color Comparison Standards.*—Arrange 9 test tubes, 16 mm. by 150 mm., in a test tube rack and pipet 9 cc. of Anstie's reagent into each tube. Then add to each tube the amounts of standard alcohol solution and distilled water shown in Table 49.

Thoroughly mix the contents of each tube by vigorous stirring with a clean dry glass rod. The standards may be kept for several weeks if tightly stoppered and kept in a vertical position in the test tube rack. The standard

solutions *must not* be allowed to come in contact with the stoppers, since both cork and rubber contain reducing substances which may cause a change in color of the standards. If the tubes can be *fire-sealed* without unduly warming the solution, the standards will be permanent for several months.

TABLE 49.—ALCOHOL STANDARDS

Tube No.	Alcohol solution (cc.)	Distilled water (cc.)	Corresponds to alcohol in the specimen (mg. per cc.)	Tube No.	Alcohol solution (cc.)	Distilled water (cc.)	Corresponds to alcohol in the specimen (mg. per cc.)
1 . . .	None	1 0	Negative	6 . . .	0 5	0 5	2 5
2 . . .	0 1	0 9	0 5	7 . . .	0 6	0 4	3 0
3 . . .	0 2	0 8	1 0	8 . . .	0 7	0 3	3 5
4 . . .	0 3	0 7	1 5	9 . . .	0 8	0 2	4 0
5 . . .	0 4	0 6	2 0				

Each standard should be labeled with the number of milligrams of alcohol to which it corresponds, *i. e.*, Tube No. 1 is labeled "Negative" or "Zero," Tube No. 2 is labeled "0.5 mg.," etc.

It is useless to try to make standards for amounts of alcohol greater than 4 mg. of alcohol per cc., since the Anstie's reagent is completely changed by that amount of alcohol. If specimens are encountered which seem to contain 4 mg. of alcohol per cc., or possibly more, run a second determination, using 2 cc. instead of 4 cc. and multiply by two to give the final reading.

2. Procedure for Blood, Urine, or Spinal Fluid.—Arrange two 25 x 200 mm tubes with two-holed rubber stoppers, and inlet and outlet tubes. The inlet tubes should extend nearly to the bottom of the tubes and the outlet tubes just below the stoppers. Connect the inlets and outlets of the two test tubes with well-washed rubber tubing in such a fashion that a current of air may be aspirated through the specimen tube into the tube of Anstie's reagent. The apparatus is shown in Figure 32.

In the specimen tube place 4 cc. of specimen, 2 to 4 cc. of Scott-Wilson reagent, and sufficient water to make 10 cc. Halfway between the upper level of the liquid contents and the bottom of the stopper, place a wad of glass wool. In the second tube place 9 cc. of Anstie's reagent. Stopper the tubes, and adjust the suction so that a current of air is aspirated through the tubes. Immerse the tubes in a gently boiling water bath. Continue boiling and aspirating for twelve to fifteen minutes. Cool the Anstie's reagent, add water until it has a volume of 10 cc., and compare its color with those of the standards. Report the result as milligrams per cc. as read from the standard matched. Each mg. per cc. corresponds to tenths of per cent.

3. Discussion.—The v
or ketosis would reduce .
indicate the apparent presence . . .

The method works well with 4 cc. of urine, but with 4 cc. of blood or of some stomach contents frothing is often excessive and troublesome, so that aspiration at a proper rate is difficult. The glass wool is used to keep the mixture of Scott-Wilson reagent and material under test from being carried over into the Anstie reagent. If glass wool is not available, add a

drop of liquid petrolatum to the reacting mixture and aspirate very slowly at first, then more rapidly. If trouble with frothing is persistently encountered, it may be necessary to use 2 cc. or even as little as 1 cc. of blood. In such a case, do not forget to multiply the apparent alcohol value obtained in matching the Anstie reagent against the standard by 2 or by 4 as the case may be. This procedure has the disadvantage that small amounts of alcohol may be missed entirely. Accordingly it may be preferable to make up the alcohol standards with 1 per cent alcohol (1.26 cc. of absolute alcohol diluted to 100 cc.) and use only 2 cc. of blood as a routine procedure.

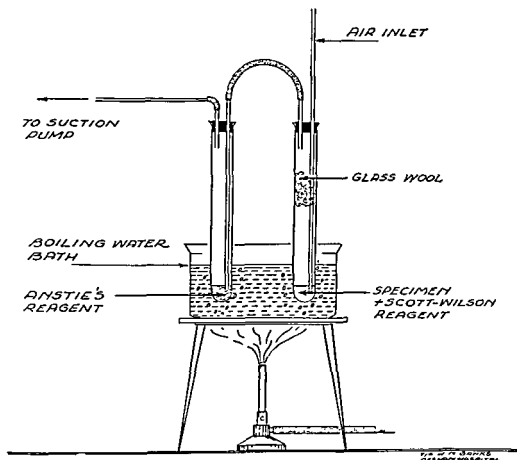


FIG. 32.—Apparatus for determination of alcohol in blood, urine, etc.

4. Interpretation of Results.—The determination of whether or not an individual has been drinking, is under the influence of liquor, or is intoxicated, is a matter for a medical officer. The amount of alcohol found in the blood or other body fluids should be regarded as *part* of the available evidence, but should not be relied upon exclusively.

A level of 0.5 mg. of alcohol per cc. of blood *may* indicate that an individual has been drinking. The result is *not* absolutely conclusive, since small amounts of reducing substances are normally present in human blood, and since slight experimental errors may occur.

At a level of 1 to 1.5 mg. of alcohol per cc. of blood an individual is usually under the influence of liquor, but not definitely intoxicated.

At a level of 2 to 2.5 mg., an individual may be regarded as definitely intoxicated.

At 3 to 3.5 mg. the individual is quite seriously intoxicated.

A level of 4 mg. of alcohol per cc. of blood indicates that an individual is either at or quite close to the "dead drunk" stage. Higher concentrations indicate very severe intoxication, which may even result fatally.

The above values are for *blood alcohol* only and should *not* be applied to urine, since the urine level indicates only the amount of alcohol being eliminated through the kidneys. The urine alcohol may be higher or lower than the blood alcohol, depending upon the stage of absorption or elimination of alcohol.

II. Lead in Urine (Method of Kaye).—Cases of suspected chronic lead poisoning are best diagnosed clinically, since individuals seem to vary in their resistance to lead. Amounts which produce definite symptoms in one person, may have relatively little effect on another. Therefore analyses for lead are mainly of corroborative value.

Analyses of feces for lead are of no significance, since the findings merely indicate the amount of lead excreted, not the amount actually present in the body. In other words, lead found in the feces indicates "ingested" lead, but not necessarily "absorbed" lead.

Unless it is desired merely to study the individual's excretion of lead under ordinary conditions of life, he should be placed on an acidotic and low-calcium diet to promote the mobilization and elimination of lead in the urine. This diet should continue for about three days before the urine sample is collected.

Since the amounts of lead found, even in severe cases of plumbism, are relatively small, and since glass, metal, and enamel vessels contain enough lead to seriously contaminate liquids placed in them, special precautions are necessary in collecting specimens of blood and urine for lead analysis.

Urine is best collected in a pyrex glass container such as an Erlenmeyer flask which has been cleaned with hot nitric acid. Add a small amount of chemically pure nitric acid to the container and heat just to boiling under a hood. Cautiously tilt the flask or bottle until the nitric acid has made contact with all surfaces, then let the vessel stand under the hood for about an hour. Rinse the walls with the nitric acid once more, then cautiously pour it off, allowing it to come in contact with all portions of the lip by rotating the vessel. Then rinse the vessel several times with distilled water. Stopper the bottle or cover the flask with an inverted beaker until used.

Collect a twenty-four-hour specimen of urine, instructing the patient to void directly into the clean bottle or flask. Measure and record the total volume and take about one-third of it for analysis.

The method is based upon the extraction of lead by means of a solution of dithizone (diphenylthiocarbazone) under carefully controlled conditions and subsequent titration of the isolated lead with a second dithizone solution.

1. Reagents.—All reagents must be lead-free or of very low lead content.

(a) *Distilled Water.*—All distilled water used must be redistilled from an all-pyrex still.

(b) *Ammonium Hydroxide.*

(c) *Nitric Acid*, 3 per cent and 1 per cent.

(d) *Potassium Cyanide*, 10 per cent solution.

(e) *Citric Acid*, 40 per cent solution.

(f) *Standard Lead*
of water. One cc. con

(g) *Dithizone 1 for 1*
thiocarbazono in 400 cc. of chloroform.

(h) *Dithizone 2 for Titration*.—Dilute 100 cc. of dithizone 1 with 100 cc. of chloroform. Standardize against the standard lead solution. One cc. equals approximately 5 micrograms of lead. The titre of this solution is satisfactory for amounts of lead near the normal. If the preliminary extraction indicates that larger amounts are present, use solution 1, each cc. of which equals approximately 10 micrograms of lead.

2. *Procedure*.—Only pyrex glassware should be used.

To the urine specimen add a few drops of phenolphthalein indicator and then ammonia dropwise until a faint but persistent pink color is produced (pH 9–10). Shake well, stopper and set aside for thirty minutes to allow the precipitate to settle. Collect the precipitated earthy phosphates and lead phosphate on a Büchner filter using suction. Wash the precipitate with several small portions of alcohol. Invert the filter paper in the Büchner funnel so the precipitate faces the perforations and pour 30 to 50 cc. of hot 3 per cent nitric acid in successive small amounts over the precipitate until it dissolves or is washed into the flask. Gently boil the redissolved precipitate for twenty minutes. Adjust the pH to 3 to 3.5 (lower range of methyl orange) by the dropwise addition of ammonium hydroxide. Transfer the solution to a Squibb separatory funnel and extract it with 5 to 10 cc. of dithizone 1, discarding the dithizone-chloroform layer. This extracts tin and bismuth and prevents later interference by these metals. At this pH lead is not extracted.

Add 15 cc. of citric acid solution and then ammonium hydroxide to pH 9 to 10, using phenolphthalein as indicator. The end-point is a faint but persistent pink. After adding 2 cc. of potassium cyanide, the solution is ready for the extraction of the lead.

Run in a few cc. of dithizone 1 and shake vigorously. The rapid appearance of a cherry-red color in the chloroform layer indicates the presence of lead, since the lead-dithizone complex which is soluble in chloroform gives it that color. Transfer the chloroform-lead-dithizone layer to another separatory funnel and again extract with a few cc. of dithizone. Continue the extractions until no more lead remains as evidenced by the dithizone solution remaining green. Wash out any remaining lead-dithizone by several extractions with chloroform, adding this also to the combined dithizone extracts.

The gradual approach to the point of complete removal of lead is shown by the diminution of intensity of the cherry-red color and the gradual change through pink, violet and blue to green. The amount of dithizone used gives a rough indication of the amount of lead present.

Shake the combined extracts with 15 cc. of 1 per cent nitric acid. The lead is now in the acid aqueous solution. Discard the dithizone layer and extract any dithizone remaining by shaking once with chloroform.

To the acid solution add a few drops of phenolphthalein and then ammonia until distinctly alkaline. Add 2 cc. of potassium cyanide solution and then the citric acid drop by drop until the solution is just faintly pink.

Titrate the prepared solution with dithizone 2. The first addition may be an amount equal to 5 cc. less than that required in the first extraction to the point where the cherry-red color began to change. Shake vigorously, allow to stratify and draw off the red dithizone layer. Further additions of dithizone are in 0.5 cc. amounts plus 1 cc. of chloroform. After each

a known lead solution by the titration as given above, the value of each cc. of dithizone in terms of lead is known. From this value and the number of cc. of solution used for the sample and the volume of the sample, the amount of lead per liter of urine may be calculated.

A blank determination should be run on the total reagents used in the titration (not in the preliminary extraction). The value of this blank, which should be low, is subtracted from the amount of dithizone used in titrating the specimen.

III. Bromides in Blood Serum.—Although the results obtained by it are only approximate, the gold chloride method is probably the simplest clinical one for determining bromides in blood. It is based upon the formation of a color, varying from a yellow greenish-brown to a strong red brown, produced when gold chloride reacts with bromide to form gold bromide. The color produced in the unknown is compared with known standards.

1. **Reagents.**—(a) *Gold Chloride*, 0.5 per cent solution.

(b) *Trichloroacetic Acid*, 20 per cent.

(c) *Sodium Chloride*, 0.6 per cent solution.—Dissolve 3 gm. of chemically pure sodium chloride in distilled water and make up to 500 cc.

(d) *Sodium Bromide-Sodium Chloride Standard Solution.*—Dissolve 1 gm. of sodium bromide in 100 to 150 cc. of the sodium chloride solution and make up to 200 cc. This solution contains 0.5 per cent NaBr and 0.6 per cent NaCl.

(e) *Comparison Standards.*—Add the standard sodium bromide-sodium chloride solution to a series of 12 test tubes, graduated at 10 cc., beginning with 0.5 cc. and increasing the quantity by 0.5 cc. in each succeeding tube. Add 0.6 per cent NaCl to each tube to the 10-cc. mark.

Place 1 cc. of each of these dilutions in a series of small test tubes, add to each 2.6 cc. of trichloroacetic acid and 0.72 cc. of the gold chloride solution and mix well.

These standards correspond to serum bromide concentrations of 25 mg. per 100 cc. in the first tube and increasing by 25 mg. in each tube to 300 mg. in the twelfth. Covered with petrolatum, stoppered and kept in the dark when not in use, these standards are quite permanent.

2. **Procedure.**—As a preliminary measure the urine may be tested. Decolorize 15 cc. of urine with activated carbon and filter. To 5 cc. of the filtrate add 1 cc. of trichloroacetic acid and 1 cc. of the gold chloride solution. If no appreciable brown color develops it is probable that the patient's blood contains little or no bromide, since excretion in the urine is quite constant and rapid.

If the preliminary urine test is positive, or history or symptoms indicate, proceed with the quantitative test in blood.

Take at least 10 cc. of blood, using all precautions to prevent hemolysis. After clotting is complete, separate the serum. Place 2.6 cc. of trichloroacetic

acid in a test tube and to it add, with shaking, 1 cc. of serum. Mix thoroughly and filter. To 1 cc. of filtrate in a tube of the same size and quality of glass as those containing the standards, add 0.2 cc. of gold chloride. Mix and compare the color with that of the standards.

3. **Results.**—Normal serum shows no change in color. Report the amount of bromides present according to the standard most closely matched.

A finding of 150 mg. of bromides per 100 cc. of serum is considered by some to indicate dangerous overdosage and to be associated with symptoms of bromidism. However, too much stress should not be placed on analytical results alone. Clinical evidence must also be considered since individuals vary widely in their reactions to the same level of blood bromide.

IV. **Quinine in Urine** (Cornell and Kaye).—This method is not specific for quinine but is of value in the military service when it is necessary to determine whether the quinine being given for malaria prophylaxis is actually being taken. Urine specimens from large groups can be tested in a short time by this procedure.

Atropine, cocaine and strychnine react to the reagent used and so may give false positives but these alkaloids would rarely be present. Albumin also interferes and would be encountered more frequently. If a positive is thought to be due to albumin heating the specimen would make the differentiation. A precipitate due to quinine will disappear on heating to reappear on cooling while one due to albumin will persist. Albumin may be removed from the urine prior to testing by heat and filtration.

Atabrine also reacts but the sensitivity of the precipitating reagent is much less for it than it is for quinine, and this in addition to the fact that both are used for the same purpose of malaria prophylaxis, makes any interference a minor consideration.

1. **Reagent.**—Dissolve 3 gm. of mercuric iodide (red), and 2 gm. of potassium iodide in a mixture of 20 cc. of glacial acetic acid and 40 cc. of water. If kept in a brown bottle this reagent is quite stable.

2. **Procedure.**—To 1 or 2 cc. of urine in a small test tube add 5 or 10 drops of the reagent. Shake.

3. **Result.**—An immediate turbidity appears if quinine is present and intensifies on standing.

V. **Analysis of Blood in Drowning Cases.**—In cases of drowning, enough water usually gets into the lungs to diffuse into the blood and be carried to the left chamber of the heart before it stops beating. Therefore in salt water drownings, the amount of chloride in the left chamber of the heart is usually increased; in fresh water drownings, the amount of chloride in the left chamber of the heart is usually decreased. If death was not due to drowning, as in cases where the body was thrown into the water after death by some other cause, the chlorides in both sides of the heart will be essentially equal.

At autopsy in cases of suspected drowning 6 cc. or more of blood should be collected from the right and from the left heart by means of pipets with wide tips. Care must be taken not to puncture the interventricular septum. Place the specimens in containers previously labeled "right heart" and "left heart" to prevent any confusion. A sample of the water in which drowning is supposed to have occurred is also collected.

The three specimens are then analyzed for chloride content by any

accurate method, such as that on page 209, and the results reported in terms of milligrams of sodium chloride per 100 cc. of blood. Normal blood contains 400 to 500 mg. of NaCl per 100 cc. Positive results are usually fairly conclusive, but negative results do not necessarily rule out drowning as the cause of death.

VI. Tablets, Capsules, and Other Medicaments.—Since many tablets and capsules are recognizable by their size, color, and general appearance, a consultation with a pharmacist will usually be helpful in preliminary identification. Tablets are usually predominantly organic or inorganic in their constituents. If inorganic substances such as sodium bicarbonate, bromides, calomel, etc., are suspected, some scheme of general inorganic analysis must be followed.

Of the tablets with predominantly organic constituents, hypodermic tablets of alkaloids are characterized by their small size. Saccharine comes in a small tablet which may be mistaken for a hypodermic tablet. The only alkaloid commonly found in capsules is codeine, usually mixed with aspirin and acetophenetidine, in the so-called "C.A.P." capsules. Caffeine is sometimes used in place of codeine. Quinine may occur in capsule, tablet or pill form, a large number of different sorts and sizes being found on the market. Most of the sulfa drugs are large, bisected, 7.7 grain (0.5 gm.) tablets. Salicylates, such as sodium salicylate, salol, and aspirin, come in 5 grain tablets, also in white capsules. Sometimes they are intermixed with other substances.

Barbiturates are usually encountered in distinctively colored capsules or occasionally in colorless tablets. The typical colors of the capsules are: Nembutal, yellow; Amytal, blue; Seconal, orange-red; Phenobarbital, white; Allonal, pink and white. Barbitol comes in a large 5-grain tablet. Amytal and Luminal may come in small 3, 1½, and ¾-grain tablets. Sodium alurate also comes in a 3-grain bisected tablet. Luminal tablets may also be bisected.

If organic
sule, powder
solution or su
it with HCl.

stand weigh the contents of the tablet or cap-

If a precipitate forms, it may indicate the presence of ...
which are insoluble in water. Add ether and extract as in working up stomach contents for organic poisons. Sulfonamides are not easily extracted from water solution by organic solvents. They can readily be detected by diazotizing and coupling a little of the aqueous solution of the tablet by the method used in determining sulfonamides in blood.

VII. Toxicological Examination of Urine.—Since the urine contains the metabolic or excretory products of most ingested poisons, it is an ideal material for preliminary testing, even if only a few cc. are available. Thus it can be used in testing for bromides, sulfonamides, barbiturates, and many other drugs and poisons. Salicy ingestion of salicylates such as sodium

6.—Cases may arise where
To 4 cc. of urine, add 1 cc.
of concentrated hydrochloric acid, and apply a few drops of the mixture to
a piece of newspaper bearing no printing. Sulfonamides will give a pro-

nounced orange-yellow color. The test is sensitive to 1 part of sulfa drug in 10,000 of urine. The reaction is due to the presence of lignin in the paper. Highly refined papers (filter paper or bond paper) will not give the test.

IX. Marihuana.—The hemp plant, *Cannabis sativa* is grown in many parts of the world for the production of hemp fiber. The various geographic

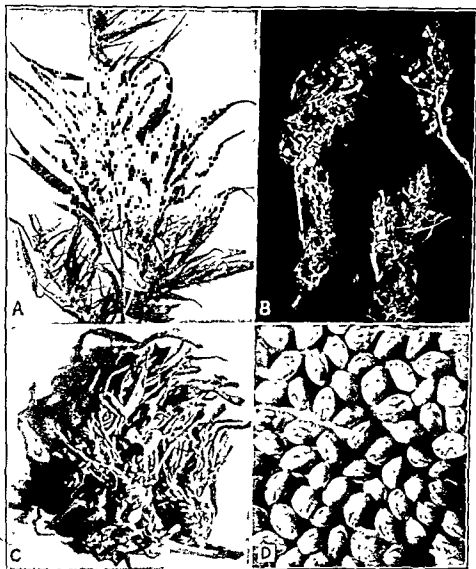


FIG 33

varieties of the plant are known as *Cannabis indica*, *Cannabis americana*, etc. The plant is sometimes used medicinally in the form of extracts, but its main importance lies in its illicit use as a narcotic drug. When thus employed, the dried plant is usually smoked in the form of cigarettes ("reefers"), sometimes admixed with tobacco. More rarely, extracts of the plant are employed.

After a little experience, rather small portions of the dried plant can

be identified readily from their appearance under the low-power microscope. If a positive identification is made microscopically, a chemical identification is usually unnecessary. For methods of microscopic identi-



FIG. 33 — Marijuana (*Cannabis sativa*). A. Female flowering top, dried, showing characteristic branching of twigs, accompanied by leaves, from main stem. Each twig supports a portion such as those shown in B and C. $\times 0.5$. B. Portions of dried female flowering tops $\times 1$. C. Portion of dried female flowering top showing branching and sub-branching of main twig and the positions of fruit and leaves. $\times 1.5$. D. Dried mature fruit without "hulls". Note the encircling ridges, the mottling and especially the lacy network covering the surface. $\times 3$. E. Dried mature fruit. $\times 17$. Ridges and lacy markings are particularly noteworthy. F. Upper side of small dried leaflet from the female flowering top. The warty appearance is due to the crystalloid hairs. $\times 17$. G. Fragments of dried leaflets from female flowering top as seen commonly in specimens submitted for examination. Note the profusion of crystalloid hair on the upper surfaces and the "wooly" appearance of the hairy undersides. $\times 16$. H. Typical sample as presented for identification. Leaves, fruit and hulls are present and are readily recognized. $\times 4$. (Marijuana, Its Identification, courtesy of the Bureau of Narcotics, U. S. Treasury Dept.)

fication see the pamphlet "Marihuana, Its Identification" issued by the Bureau of Narcotics, U. S. Treasury Dept.

The illustrations in Figure 33 are a few of the many in this booklet. Identification of unknown specimens should be checked against a sample known to be marihuana.

The chemical identification of suspected extracts of the plant is much more difficult, and all of such materials should be referred to a toxicological laboratory.

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CHAPTER XX

USEFUL LABORATORY ARTS AND RECIPES

By WALTER C. TOBIE

OF the many aids and recipes so generally useful in all laboratories, only a small number can be included here. Those selected are the simpler ones of wide applicability; for the more elaborate ones the reader is referred to the list of texts at the end of this chapter.

I. Handling and Working Glass.—1. **General.**—Although many different grades of glassware are available, they may be roughly classified as soft glass and hard or pyrex glass. Soft glass tubing, rods, and test tubes of small diameter are easily manipulated with an ordinary Bunsen or Tirrill burner, with or without a fishtail or wing-top attachment. Larger diameters require the use of a blast burner with compressed air. Hard glass such as pyrex can hardly be worked at all without a blast burner. For handling large pieces of pyrex, an oxygen blast must be used. The paragraphs which follow refer to the bending of soft glass tubing. Methods of handling pyrex are given in reference No. 1.

2. **Cutting Glass.**—Hold the glass tube or rod firmly on the top of a laboratory table and nick it at one spot with the edge of a triangular file. Then take the tube or rod in both hands, with thumbs opposite the nick, exert a slight pull on the tubing and break with a quick snap. If one end of the tubing is too short to handle, the snap may be made by holding the long end rigidly in one hand and hitting the small end with the file. The sharp end of the tube or rod is smoothed (fire-polished) by heating it to redness in the flame. If desired, the bore may be reduced by overmelting or increased in size by manipulating with the end of the file.

3. **Cutting Test Tubes.**—Make a deeper file nick than with glass tubing and preferably encircle the tube. A thin tube may be broken by hand as previously described. Thick tubes should be nicked even more deeply, and a red-hot nail applied to the nick to start a crack around the tube. It is preferable *not* to heat the handle of the file for starting the crack since such treatment will ruin its temper. Cutting test tubes or plain glass tubing of more than 10 mm. diameter is best done with an electrically heated resistance wire wound around the tube at the place to be cut, if such a device is available. In any case, the sharp end should be fire-polished.

4. **Bending Glass Tubing.**—Holding both ends, slowly rotate the tubing in a hot flame using a wing-top attachment if available, so that at least an inch is heated. When the glass is red hot and soft, remove from the flame and bend to the desired form. For a broad bend, several inches should be so heated. If the tube is overheated, there is a tendency to pull the ends of the tube apart, and for the red hot glass to distort by its own weight. If underheated, the bend will be very sharp with the glass buckled out laterally. A satisfactory bend retains essentially the same diameter as the original tubing. If the tube collapses for any reason, it can be brought to proper diameter by heating the collapsed portion and applying air

pressure by mouth to one end, while holding the other end closed with the finger. This procedure requires considerable practice.

5. *Drawing-out Glass Tubing.*—To draw out glass tubing, heat the selected portion red hot while gently rotating the tube, remove from the flame and pull the ends apart. For a short taper, be careful not to over-heat and to pull apart slowly. Strong heating and rapid pulling results in a very long, thin, capillary tube.

6. *Ampules.*—Heat a clean, sterile, cotton-plugged test tube about 2 inches from the mouth, with constant rotation. When the glass is dull red, draw the two ends about 2 inches farther apart, leaving a neck about 4 mm. in diameter. In filling the ampule, be careful to get no liquid on the neck. Heat the neck in a small, hot flame until the glass collapses, then pull out rapidly. If a strand of glass protrudes, heat it rapidly in a hot flame until it melts down to a small bead. A large bead will usually crack off on cooling. In sealing commercial ampules, which are already drawn down to a taper neck, it is best to heat with a small hot flame about 3 or 4 mm. below the mouth, until the neck becomes red hot and thickens. Then rapidly draw out the hot glass with a pair of forceps. If necessary, seal the extremity to form a small bead as before. Do not heat too much or the warmed air inside will expand and blow a hole in the molten glass.

7. *Repairing Damaged Glassware.*—Many pieces of damaged glassware can be repaired and rendered usable again. Pipets cracked at the mouth-piece can be fire-polished directly or after a short piece has been cut off. If the tip is broken, it can be drawn out again, but such pipets should be marked to show that they no longer are suitable for exact measurements. Beakers and flasks with chipped edges can be repaired by fire-polishing, but since such apparatus is usually of pyrex, a blast flame is necessary. Burets and cylinders cracked at the mouth can be shortened by holding them in the hand and chipping away the broken portion by rapidly stroking or striking it at an angle with a square of wire gauze, until the new neck is approximately even, after which it may be fire-polished.

8. *Glass Tubes in Cork or Rubber Stoppers.*—Glass tubing passed through holes in cork or rubber stoppers should be of such diameter as to fit tightly without requiring undue force in its insertion. In cutting a hole in a stopper, select a cork borer of a size just a trifle smaller than the glass tube which is to be inserted in the hole. Cork borers must be kept well sharpened. In cutting holes in stoppers and in passing glass tubing through the holes it is often well to use a little water or glycerol as a lubricant. If a glass tube becomes stuck in a stopper, it may sometimes be loosened by sliding a cork borer over it and into the stopper so as to break the adhesion. Another method is to put the short end of the tube against some firm surface and to press down on the stopper, twisting it with the hands to loosen it from the glass. Sometimes a slim knife blade can be thrust between the glass and the stopper. If these efforts fail, cut the stopper longitudinally with a sharp knife or razor blade and peel the stopper off the glass.

II. *Cleaning Glassware.*—1. *Mechanical Cleaning.*—Glassware can be perfectly cleansed with soap, water, and a brush in many cases. Cleaning is much easier if the glassware is immersed in water immediately after use, so that solid matter does not dry on it. Test tube brushes will have a longer useful life if they are washed free of soap and are allowed to dry when not in use. Dirt in flasks or bottles which cannot be reached with a

brush can often be loosened by adding fine shot and swirling or shaking the apparatus.

2. *Chemical Cleaning Solutions.*—(a) *Trisodium Phosphate.*—This is a strongly alkaline salt which is an ingredient of many cleaning preparations. It is excellent for removing greasy films from glassware, which may be soaked in a 5 to 10 per cent solution for fifteen to thirty minutes, scrubbed with a stiff brush, then rinsed and dried. It has the disadvantage of some-
in it. It should

(b) *Nitric Acid.*—Hot nitric acid is extremely effective in oxidizing organic matter. It must be used with due caution to avoid spilling on the hands and clothing and to avoid breathing the rather toxic vapors. Immerse the glassware in a beaker of nitric acid under a hood, heat just to boiling and let cool.

(c) *Sulfuric and Nitric Acid Mixture.*—This is probably the best preparation for the routine cleaning of glassware containing much organic matter. A large porcelain evaporating dish or other acid-resisting container filled with sulfuric acid is placed on a gas range or electric hot-plate under a hood. A small amount of nitric acid is stirred in well at the beginning of each day's work, before turning on the heat; otherwise, it will form a layer on the surface and will evaporate rapidly as the bath becomes hot. This cleaning bath may be maintained for several months, and need only be discarded when the sulfuric acid has become viscid by the slow accumulation of inorganic salts from the oxidized materials. The bath should not be heated so hot that white fumes of sulfuric acid arise. Organic matter at first causes the bath to turn brown, but when it is completely oxidized, the bath becomes nearly colorless again, so that small objects are easily picked out of it with a crucible tongs. If a porcelain evaporating dish on a tripod is used, the dish should be reinforced by placing a coarse wire screen under it. If the mixture is heated in a "Duriron" dish, mechanical breakage of the container need not be feared. The 16½-inch size (obtainable from the Duriron Co. of Dayton, Ohio) is large enough for most laboratory glassware. Traces of grease on pipets may be removed by a few hours immersion in cold sulfuric acid containing about 10 per cent by volume of nitric acid.

(d) *Sulfuric and Chromic Acid Mixture.*—This is used either hot or cold, and is prepared by saturating sulfuric acid with sodium or potassium dichromate (or better, chromium trioxide). A liter of concentrated sulfuric acid may be poured into 35 cc. of a saturated solution of technical sodium dichromate. When freshly prepared, this is an excellent cleaning solution, but it rapidly becomes spent if much organic matter is oxidized, turning green by the reduction of chromium trioxide. More of the chromium compound may be added, but the mixture soon becomes so thick and pasty that it must be discarded. It has the further disadvantage of having a very dark color, so that it is extremely difficult to find small objects and to remove them from the bath. Since traces of chromium have a strongly toxic action on many microorganisms, glassware, especially fritted glass filters, after immersion in this cleaning solution, must be rinsed with extreme care before bacteriological use.

III. *Stopcock Lubricants.*—1. *Commercial Lubricants.*—The most convenient stopcock lubricants are those sold commercially in tubes or jars.

Many different preparations are sold under proprietary names, but practically all are satisfactory for the use for which they are intended.

2. **Laboratory-made Lubricants.**—The following lubricants may be used when commercial lubricants are not available. The gum rubber which is called for may be cut from the soles of an old crepe-soled shoe. Other forms of rubber, containing filler, are much less satisfactory.

(a) Melt 2 parts of paraffin by weight and 4 parts of petrolatum (petroleum jelly) together, and add slowly 1 part of gum rubber cut into small pieces. Stir while heating until a smooth paste is formed. Be careful not to overheat and burn the rubber.

(b) Mix 10 parts by weight of petrolatum and 1 part of gum rubber cut into small pieces. Hold at 125° to 150° C. in an oven for several days, with occasional stirring, until the paste is smooth.

(c) Mix 2 parts by volume of ordinary rubber cement and 1 part of petrolatum. Heat on a water bath until the solvent from the rubber cement has been driven off.

3. **Petrolatum.**—Petrolatum jelly is a fairly good stopcock lubricant when nothing better is at hand, but is rather thin-bodied and likely to permit the freezing of stopcocks. Liquid petrolatum may also be used but is even thinner bodied.

4. **Glycerol.**—The lubricants mentioned above are all dissolved to some extent by organic solvents, such as ether, petroleum ether, chloroform, etc. For sealing ground-glass joints and stopcocks against substances which do not dissolve it, glycerol is a good lubricant. It should not be used with alcohol, in which it is soluble.

5. **Glycerol-Starch.**—Suspend 9 gm. of soluble starch in 22 gm. of glycerol and heat to 140° C., stirring with a thermometer. Pour off the clear liquid and let it cool. This lubricant is insoluble in organic solvents except alcohol, and is fairly resistant to the action of water, so that it is useful on the stopcocks of separatory funnels in extractions in which aqueous solutions are shaken with some immiscible solvent.

IV. **Cements and Adhesives.**—1. **Vacuum Wax.**—For ordinary vacuum seals, and for vacuum distillations where the temperature does not go too high, an excellent wax may be made by melting together equal parts of beeswax and rosin. The wax is pliable and easily removed by using hot water. Ordinary stopcock lubricants can be used in place of vacuum wax in many cases.

2. **Acid-resisting Cement.**—Mix asbestos powder and sodium silicate solution ("water glass") to a thin paste. If allowed to dry for a day, this cement will resist strong acids. A thick paste of asbestos mixed with water containing a little sodium silicate solution makes an excellent insulating

frozen joints can usually be loosened by appropriate means. The process requires patience. "Strong arm" methods will usually result in breakage, and the loss of the apparatus.

1. **Commercial Mechanical Devices.**—There are a number of excellent devices on the market for loosening glass joints. Since they are somewhat

expensive, it is usually worth-while to purchase them only if they can be used by a considerable group of laboratories. Their successful use is based upon the *gradual* application of pressure or tension to the glass joint, care being taken that the devices are not allowed to slip and fracture the glass.

2. **Laboratory-made Mechanical Devices.**—By a study of the principles upon which the commercial devices operate, it is sometimes possible to improvise a mechanical device which will serve the same purpose. For example, a piece of wood may be drilled and cut in such a fashion as to furnish a support for a glass joint which is inserted in a vise and *gentle* ruding end of a stopcock. Glass stoppers in by gentle tapping with the *butt* of a hammer or similar tool.

3. **Use of Heat.**—The proper application of heat alone will loosen many frozen glass joints. Chill the joint in an ice bath, then immerse it suddenly in warm or hot water. This will cause the exterior portion of the joint to expand away from the stopcock or glass stopper, so that it may be loosened by hand or by other means.

4. **Chemical Methods.**—Ground-glass joints frozen by contact with alkali can frequently be loosened by immersion in a dilute (about 5 per cent) solution of acetic or hydrochloric acid which is brought just to a boil, then allowed to cool so that the dilute acid penetrates the joint as it contracts. It may be necessary to heat and cool several times. In stubborn cases, several days may be required. If a joint contains lubricant or other organic material, it may be loosened in the same fashion in a bath of concentrated sulfuric acid. Large pieces of apparatus, such as burets, should be supported by means of a clamp or a ring on a ring-stand during the process.

VI. **Grinding and Fitting Stoppers and Stopcocks.**—Glass stoppers can be ground to fit bottles, volumetric flasks, etc., from which the original stopper has been broken or lost, as follows: Select another glass stopper which is a little too large but which makes a fairly close fit in the neck. Apply a thin mixture of glycerol and emery of medium fineness to the new stopper and rotate it in the bottle or flask neck steadily in one direction. As the cutting mixture oozes out, apply more and continue the process. When the stopper is well ground in, wash off the cutting mixture with water. The stopper should now make a tight fit without wobbling in the neck. If desired, a better fit can be obtained by finishing the grinding process with a finer grade of emery. This process works well in fitting glass stoppers to bottles and flasks. It may also be employed, using fine emery, to dress buret stopcocks which have been damaged by contact with alkali, but care must be taken not to continue the grinding to such a point that the hole in the stopcock will no longer line up with the passage through the buret.

VII. **Marking and Labeling Glass.**—1. **Glass-marking Crayons.**—For temporary markings on glass, use the special crayons or pencils manufactured for the purpose. These may be obtained in various colors. In order to write distinctly with them, the glass must be free of any traces of oily material. Red and blue glass-marking crayons are furnished as Items 76290 and 76300 of the Medical Department Supply Catalog.

2. **Gummed Labels.**—For semi-permanent markings on glass containers, gummed labels should be used. Complete information should be given on the label as to what the material is, its strength, date prepared, by whom prepared, and purpose for which prepared. Typed labels are best. Much confusion and inconvenience is caused by failure to properly label containers. Labels may be protected against most reagents by coating them with collodion or vinylite lacquer. Such labels are best removed from bottles by scraping with a sharp knife or razor blade.

(a) *Collodion for Labels.*—Dissolve 3 to 4 gm. of pyroxylin in 25 cc. of absolute ethyl alcohol, then add 75 cc. of ether.

(b) *Vinylite Lacquer.*—Dissolve 20 gm. of vinyl acetate polymer (Vinylite A) in 100 cc. of solvent made by mixing 75 cc. of toluene and 25 cc. of 95 per cent alcohol. This lacquer gives a transparent, colorless film which is resistant to most reagents.

3. **Diamond-tipped Pencil.**—For absolutely permanent markings (as for identification of glassware, or in graduating apparatus) use a diamond-tipped pencil such as Item 42100 of the Medical Department Supply Catalog. If the pencil does not mark or scratch the glass, rotate it slightly in the hand so as to bring one of the facets into proper position, when it will be found to mark the glass clearly and distinctly.

VIII. Acidproof Wood Stain.—

Solution No. 1		Solution No. 2	
Copper sulfate . . .	125 gm.	Aniline (technical) . .	150 gm.
Potassium chlorate . .	125 gm.	Hydrochloric acid . .	180 gm.
Water	1000 cc.	Water	1000 cc.

The wood of the bench or table top should be free of paint, varnish, grease, and chemicals. With a paint brush, apply two coats of solution No. 1 boiling hot, allowing each coat to dry completely before applying the next coat. Then apply two coats of solution No. 2 in the same way. When the wood is completely dry, wash off all excess chemicals with hot water and soap. Scrub until no more color comes away. Dry, and rub in raw linseed oil with a cloth or sponge.
resistant to most acids and other chemicals.
dingy, go over it again with linseed oil and
oil by rubbing with a cloth or paper towel.

IX. **Removal of Stains.**—1. **Bacteriological Stains.**—Most bacteriological stains can be removed from the hands by washing with acid alcohol (ordinary ethyl alcohol containing 2 or 3 per cent by volume of concentrated hydrochloric acid), then washing with soap and water. For cleaning fabrics, use 10 per cent by volume of acetic acid in alcohol and rinse with large amounts of water. If some color persists, treat with dilute chlorine or bromine water or filtered solution of chlorinated lime (such as "HTH," high test hypochlorite) and rinse again.

2. **Iodine Stains.**—These are best removed with a solution of sodium thiosulfate, followed by rinsing.

3. **Bloodstains.**—These are removed by applying 3 per cent hydrogen peroxide, followed by rinsing.

4. **Silver Stains.**—For stains due to silver compounds such as silver nitrate or argyrol (silver proteinate, protein silver), use a hot solution of 5 gm. of mercuric chloride and 5 gm. of ammonium chloride in 100 cc. of water.

5. **Mercurochrome Stains.**—Fresh mercurochrome stains can be removed with dilute chlorine water, bromine water, or a filtered solution of chlorinated lime (HTH, high test hypochlorite). Old mercurochrome stains should be treated with a 2 per cent solution of potassium permanganate, followed by oxalic acid to remove manganese dioxide stains from the fabric.

6. **Biological Fluids.**—The stains and odor of putrefaction from biological fluids can be removed from the hands or from fabrics with permanganate solution, followed by oxalic acid solution, and thorough rinsing.

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PART III

Mycology

CHAPTER XXI

GENERAL MYCOLOGICAL TECHNIC

BY VIRGIL H. CORNELL

MEDICAL mycology has been generally restricted to the study of those fungi causing disease or of the contaminants encountered incidentally during efforts to recover such pathogens. Limited knowledge of the broader fields of mycology has led to confusion among medical laboratory workers, and is the reason for the lack of interest and the unsatisfactory results usually associated with mycological examinations.

When fungi are found in human or animal disease they must not be too readily accepted as etiological agents. An effort should be made to identify them as belonging to a genus known to be responsible for disease of the type encountered and, when possible, the organisms should be inoculated into animals to determine their pathogenicity. Certain fungi may not be pathogenic in the ordinary sense of the term but, by their presence and continued growth, may so complicate a disease condition that their eradication is desirable.

In the following brief notes an effort has been made to outline procedures for the routine identification of pathogenic fungi. A definite classification of fungi is not attempted and some of the terms are not mycologically correct but are commonly used.

METHODS OF EXAMINATION

1. *Microscopic Examination.*—1. *Materials.*—For routine use the following items should be kept on hand: Glass slides, 3 inches by 1 inch; also a few 3 inches by 2 inches; cover slips, preferably square; hollow-ground slides; fine forceps, pointed; fine scissors, pointed; safety-razor blades; platinum and one spatulate; . . . hydroxide, and the . . . cent; sterile saline; 100-cc. flasks; sterile Petri dishes; a 2-cc. syringe of vaseline, with needle; dropping bottle of lactophenol (lactic acid, 20 gm.; phenol crystals, 20 gm.; glycerol, 40 gm.; distilled water, 20 gm.).

2. *Collection of Specimens.*—In this type of work it is advisable that the specimens be collected by the individual who is to make the examination. When possible, have the patient report at the laboratory, but if this is inconvenient, secure the specimens on the ward or in a dressing room. After cleansing the affected part with 75 to 95 per cent alcohol or with

sterile salt solution, such infected materials as hairs, nails, scales or bits of tissue, may be collected and placed in a sterile Petri dish. If the material collected consists of scrapings from moist surfaces, washings from irrigated sinuses or small biopsy specimens, precautions must be taken to prevent drying and contamination. Moist preparations should be prepared and examined without delay. Biopsy specimens should be divided, one-half for mycological examinations and the remainder for fixation and histological examination.

In all cases try to obtain the specimen from an actively infected area and not from dried or inactive lesions. Sinuses may be gently irrigated with sterile saline to cleanse them, and then somewhat more forcefully to dislodge the material for examination. Curettement of sinus walls or the borders of the infected areas may yield the best samples. A sterile safety-razor blade is useful for obtaining small bits of tissue from intact lesions.

Before collecting sputum the teeth should be well cleansed and the mouth rinsed with 25 per cent alcohol or sterile saline. The specimen is taken in a sterile Petri dish and promptly delivered to the laboratory. Do not use specimens collected over a period of several hours for this purpose.

3. Preparation of Specimens for Examination.—When examining moist materials from ulcers, granulomas, sinuses, abscesses, or similar lesions, make smear preparations of the exudate, pus or crushed bits of tissue as for bacteriological study, including additional smears to be stained by the Giemsa and Wright methods. Such stains not only assist in the study of any fungi present but also bring out cellular detail and may lead to the discovery of a protozoan infection, such as leishmaniasis.

The curettings, pus, hairs, scales, and the material mentioned above, should also be prepared as follows, for mycological study: Outline a vaseline square or circle on a clean slide, by gently warming the needle of a 2-cc. syringe containing vaseline and tracing the design; slight pressure on the plunger is all that is needed for an adequate supply. Place the material to be examined in the center of the ring or square and add 1 or 2 drops of the 10 per cent sodium hydroxide. Some workers prefer a 20 per cent potassium hydroxide for this purpose. Cover the preparation with a clean cover slip. After a period of digestion the preparation is ready for examination. This period will vary with the density of the material examined, at times requiring only a few moments but on other occasions twelve to twenty-four hours. Heating the preparation over a low flame of a Bunsen burner or alcohol lamp allows immediate clearing and avoids the delay of twelve hours. This preparation, when cool, can be rimmed with vaseline to avoid drying. It is advisable to establish a routine of examining these preparations immediately after completing the cultural work and again the next morning. Fungi resist the digestive action of the hydroxide and retain their form, whereas the tissue elements disappear. Avoid mistaking artifacts resembling yeast-like organisms and hyphae. They will not be seen either in similar preparations made with saline or in stained smears and such can be used as check methods until the examiner becomes more familiar with these artifacts. The epithelial cells show at times a separation of their borders which resembles fungi and is sometimes referred to as "mosaic."

II. Cultivation.—1. Media.

(a) *Sabouraud's Maltose Agar.*

Peptone	10 gm.
Maltose	40 "
Agar	15 "
Water	1000 cc.

(b) *Sabouraud's Conservation Medium.*

Peptone	30 gm.
Agar	15 "
Water	1000 cc.

(The pH of these two media should be correct without titration, i. e., between 5 and 6. More agar may be used, giving a stronger surface.)

(c) *Cornmeal Agar.*—Heat 62.5 gm. of cornmeal in 1500 cc. water for one hour at 60° C. Filter through paper; adjust volume to 1500 cc. Add 19 gm. of agar and heat in an Arnold sterilizer an hour and a quarter. Filter through cotton, tube and sterilize; tubes for slants or plates should be made. The pH will be correct without adjustment.

(d) *Czapek's Medium* (Modified by Dox and by Thom).

Sucrose	30.00 gm.
Sodium nitrate	2.00 "
Dibasic potassium phosphate	1.00 "
Magnesium sulfate (crystals)	0.50 "
Potassium chloride	0.50 "
Ferrous sulfate	0.01 "
Water	1000.00 cc.

(Agar may be added to form solid medium)

(e) Various enrichment substances have proved useful and may be used, such as glucose (1 to 4 per cent), honey (8 per cent), carrot infusion, potato infusion, soluble starch (0.2 per cent), beef infusion, beef extract, veal infusion, brain extract, spleen extract, and the various sugars. Koser and Saunders¹ recommend the use of mannitol for *Streptothrix* and the vitamin B complex for *Streptothrix* and *Saccharomyces*, which is also of value in media for *Torula*. They give results of experiments with various substances as aids to growth.

The yeast-like fungi *Candida* (Monilia) can be identified easily by the methods of Martin, *et al.*² These methods include the following: (1) The fungus is isolated on Sabouraud's glucose agar slant, transplanted to (2) Sabouraud's glucose acid broth and incubated at 37° C. for forty-eight hours. The tube is then shaken in a beef-extract medium for ten days, the type of colony is noted, and (4) a well-isolated colony is picked and transplanted to a Sabouraud's glucose agar slant. This is incubated at room temperature or 37° C. for twenty-four to forty-eight hours. Some of the growth is transplanted to a carrot plug which is kept at room temperature and subsequently examined for asci. (5) The rest of the material is streaked on

sterile chamber for several days. The slide is then fixed, stained and examined microscopically for details of mycelial growth. (7) Four beef-

extract broth tubes, containing 1 per cent of glucose, sucrose, lactose and maltose respectively, are inoculated with a pipet containing a saline suspension of the last transplant of the fungus on the beef-extract agar slant.

Since *Candida* (*Monilia*) *albicans* is the only pathogenic member of this group, the identification of this species is important. When the culture has been purified, streak with a straight inoculating wire through a cornmeal agar plate making a slight furrow. The fungus will produce mycelium at right angles to this furrow and by inverting the plate and using the low-power objective the growth can be examined microscopically. The appearance of characteristic chlamydospores is diagnostic. As a further test—1 cc. of a 1 per cent suspension of *C. albicans* injected (intravenously) into a rabbit kills this animal in four to five days. Typical kidney lesions should be noted at postmortem examination of the rabbit.

Actinomyces should be cultured in veal infusion dextrose (pH 7.6) shake cultures at 37° C. Such cultures provide micro-aërophilic conditions necessary for the anaërobic *A. bovis*. This material should also be cultured on Sabouraud's or beef infusion agar at 37° C. and room temperature since some pathogenic species of *Actinomyces* (*A. asteroides*) are aërobic.

Incubator temperature and blood agar frequently produce the growth of the yeast-like tissue forms of some fungi that develop filamentous cultures on artificial media at room temperature. *Blastomyces dermatitidis*, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis* can be converted to the tissue form or maintained in the tissue form by culturing at 37° C.

2. *Technic*.—Scales, hairs, fragments of tissue, pus, centrifugalized fluids, etc., should be placed upon media having a pH of 5 to 6. Inoculate Sabouraud's maltose agar slants with the material using 4 or more tubes for each culture and making 3 or more plants on each tube. Inoculate the slants so that the surface of the medium is slightly broken and the material is introduced just beneath the surface of the medium. Incubate at room temperature and in an incubator at 22° C. Some fungi, if highly parasitic, grow better at 37° C. Many show differences in their cultural

characteristics at 22° C. and 37° C. hence, incubation of
Retain the cultures
for at least four weeks before discarding. Due to the
slow growth it is advisable to protect from excess evaporation either by
using rubber caps on the tubes or by placing tubes and plates in some
form of moist chamber.

Observe the cultures daily, but do not open the tubes unless definite growth is observed; then make at least one subculture as soon as the tube is opened. Subcultures are made on Sabouraud's maltose agar or Sabouraud's conservation medium. The former is best for primary isolation; the latter for storage or for slower growth and the study of certain characteristics. Several subcultures on various media from the first growth are always advisable in order that some may remain unopened while others are studied, and to assure growth on that most favorable to the organism recovered.

Various methods for staining fungi in tissue and in cultures have been described. In tissue, the routine histologic methods are adequate. For cultures, the filamentous fungus should be picked from the medium with a slightly bent inoculating wire. This material should be placed in a

drop of lactophenol to which cotton blue stain has been added and then teased with dissecting needles. The well spread material is then covered and yeast-like fungi should

different details; a subdued light, obtained by lowering the substage (with low-power objective) and use of the diaphragm, is usually best. Study budding, sporulation, etc., before a dilute dye may be run under the cover Even tube cultures may be well observed

under the low power of the microscope by viewing the edge of the slant. Hanging-drop cultures or drops of maltose agar placed on a sterile slide, inoculated and covered with a sterile cover slip, afford another means of cultural study. It will be found useful to make numerous sketches, true as to details, for later study and comparison. Thus, differences between cultures and between closely-related species will be recorded and noted. It cannot be too strongly stressed that this will also familiarize the worker with details otherwise easily overlooked.

III. Animal Inoculation.—The mouse, guinea pig or rabbit may be used to test the pathogenicity of isolated fungi. Intracutaneous, subcutaneous, intravenous or intraperitoneal injection routes may be used. Certain yeast-like organisms which produce focal lesions can thus be obtained in pure culture. However, many of the fungi pathogenic for man fail to infect such animals. Some fungi which produce lesions after injection by the intravenous route are innocuous when inoculated by other routes.

DEFINITIONS

Thallus: The actively growing, vegetative organism as distinguished from the reproductive portions.

Hypha: The single thread-like portion.

Mycelium:

Septa:

Spores:

Conidia:

budding or septate division.

Conidiophore: The hypha bearing a spore or group of spores.

Ascospores: Group of spores, usually 4 or 8, enclosed in a sac, or ascus.

Oospores and Zygosporangia: The spore resulting from the union of two similar spores is a zygospore; if the spores uniting are male (antheridium) and female (oosporangium), the resultant spore is an oospore.

Endospore: A spore formed within an outer envelope.

Blastospore: A spore formed by budding.

ments of a hypha and released by

Chlamydospore: A large spore, either intercalary or terminal, with tough and frequently double contoured (thick) wall, undergoing encystment.

Thallospore: Any spore formed from the main hypha (or thallus) directly, as in the preceding three.

Sterigma:

Vesicle:

Columella: The distal end of a hypha forming the supporting center of a sporangium.

Coremium: Bunched groups of conidiophores seen in some species. (Resembles a bunch of asparagus.)

Sporangium: A sac containing an indefinite number of spores, usually many, at the end of a hypha.

Sporangiophore: A hypha bearing a sporangium.

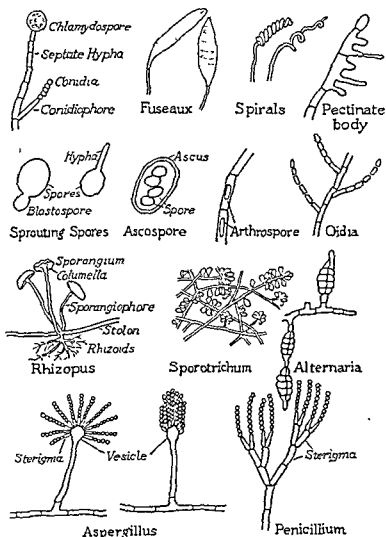


FIG. 34

Stolon: Runner-like branches of certain fungi (Rhizopus).

Rhizoids: Root-like groups occurring along stolons.

Fuseaux: Fusiform septate spores, produced by certain skin fungi (Trichophyton).

Spirals: Terminal coils seen in some species of skin fungi.

Pectinate Bodies: Comb-like structures formed by some skin fungi.

IDENTIFICATION AND CLASSIFICATION

Purity of culture is of prime importance. This can be secured only by careful technic and repeated subculture. Inoculation of Sabouraud or

TABLE 50

Vegetable kingdom	1. Spermatophyta (seed plants)	A. Algae (chlorophyll)	1. Schizomycetes (bacteria)
	2. Pterydophyta (fern plants)		
	3. Bryophyta (moss plants)		
	4. Thallophyta (thallus plants)	B. Fungi (no chlorophyll)	2. Eumycetes (true fungi)
			3. Myxomycetes (slime molds)

TABLE 51

Eumycetes (true fungi)	1. Phycomycetes (non-septate mycelium, zygospores present).
	2. Ascomycetes (ascospore production characteristic but may not be seen; conidia produced and also asexual reproduction, mycelia septate).
	3. Basidiomycetes (basidia, or club-like hyphae bearing spores; non-pathogenic).
	4. Hyphomycetes (fungi imperfecti). (Septate mycelium; free-born spores or conidia)

NOTE: In these tables the organisms of greater importance in medicine are indicated by use of boldfaced type.

TABLE 52

CLASS	ORDER	FAMILY	GENUS
1. Phycomycetes	Mucorales	Mucoraceae	Mucor
			Bisomycor
	Saccharomycetales	Saccharomycetaceae	Saccharomyces
			Schizosaccharomyces
2. Ascomycetes	Aspergillales	Aspergillaceae	Aspergillus
			Monospora (etc.)
	Endomycetales	Endomycetaceae	Endomyces
			Coccidioides
3. Basidiomycetes—non-pathogenic (rusts, smuts, mushrooms, etc.)	Gymnosporiales	Gymnosporaceae	Trichophyton
			Microsporum
	Aspergillales	Aspergillaceae	Acheris
			Epidermophyton
4. Hyphomycetes—true and imperfect (Fungi imperfecti)	Heterosporiales	Heterosporaceae	Penicillium
			Aspergillus
	Heterosporiales	Heterosporaceae	Trichosporon
			Trichosporon
5. Basidiomycetes—pathogenic (Fungi imperfecti)	Heterosporiales	Heterosporaceae	Heterosporon
			Heterosporon
	Heterosporiales	Heterosporaceae	Heterosporon
			Heterosporon

cornmeal agar plates and the transfer of isolated colonies to tubed media is, perhaps, the best method. Broth, gelatin, potato, various sugar media and at times other special media, are used. Nearly all fungus cultures when first grown contain some bacteria. These must be eliminated before fermentation tests are performed and stained smears of all positive tubes must be studied to assure purity of culture. The use of low pH, lower incubator temperatures or certain mild antiseptics may inhibit the bacterial growth. Infusion broth tubes inoculated with fungi and showing no general clouding in forty-eight hours when incubated at 37° C., are probably free from bacterial contamination. The fungi show more variation in response to environment than do most bacteria. Hence, it is important that the type of growth be always considered in conjunction with the media used.

As in bacteriology these growth characteristics on certain media are the basis of classification together with biologic characteristics, dependent on the presence of sugars, the oxygen tension, liquefaction or coagulation of certain substances, etc. For these criteria reference must be made to texts on mycology and recent articles on some groups, such as the yeasts. Classification of fungi is a task too complicated and time-consuming to be performed by most clinical pathologists, but the characteristics of the more common pathogens and contaminants can readily be learned. Some idea of the place of these organisms in the scheme of life may be obtained from the accompanying brief outline.

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CHAPTER XXII

DIAGNOSIS OF FUNGUS DISEASES

By VIRGIL H. CORNELL

COMMON SKIN INFECTIONS

DEPENDING on the type of lesion and the anatomical region involved, one should examine hairs, scales, tops of vesicles, curettings from borders of ulcers or sinuses, purulent discharge or portions of biopsy specimens, both by direct microscopic study in sodium hydrate solution and by culture. Biopsy specimens should be divided for mycological and histological studies before fixation.

In cases of *tinea capitis* the hairs will show round or polygonal spores in mosaic form, about the shaft of the hair; and, occasionally, some hyphae in adjacent scales. Cultures will produce a white, then gray, felt-like growth, and frequently conidia, fuseaux, pectinate bodies and fusiform bodies. Some produce a yellow pigment. In other cases microscopic study will show the spores in closely packed long chains within the hair and culture produces white to yellow downy colonies composed of mycelium and spores. Classification depends upon the form and location of the fungus in or on the hair and upon the type of culture produced. It suffices usually to identify an unmistakable mass of spores within the hair or upon it. The ophyta. The separates from not the hair. ---

coated with spores with perhaps a few hyphae in the adjacent epidermis, the fungus may be tentatively classed as a *Microsporum*. If there are many hyphae and spores in the hair with some in the epidermis, it is likely a

clinical]

From

by the *Microsporum*, ---

lesions are not typical.

In *erythrasma* or *pityriasis versicolor* scales of skin removed from the advancing edges of the lesions may show some mycelia and numerous spores, but cultural results are generally negative.

In many cases of *tinea cruris* or similar eruptions of the neck, axilla, body and limbs, and in the *tinea* lesions of hands and feet (including nails), there are found branching septate hyphae. Avoid being misled by anastomosing lines of cleavage between squamous epithelial cells. Culture will yield downy growths, generally white in the early stages but occasionally turning pink, brown, gray or black, in part, or producing such pigments in the medium beneath. On these and other characteristics depends the ultimate classification. Some species form peculiar end organs such as fuseaux, spirals, pectinate bodies, etc., which, when found, make the

diagnosis more certain. The lesions of "athlete's foot" and "dhobie itch" are due to fungi of this group. The general term dermatophytosis may be applied and the fungus reported as *Trichophyton* or *Epidermophyton*.

Papular or even vesicular lesions may be seen on the hands, face and

an allergic reaction to the fungus or its products.

Lewis, MacKee and Hopper⁸ report on the intracutaneous test with trichophytin as observed in over 1000 patients. They consider the test specific, this specificity being for the genus rather than for species. They conclude that certain species may not sensitize; that the patch test is not as efficient and that a negative result in the presence of a proved fungous infection by this genus indicates a poor prognosis. A positive test under such circumstances is considered as being a favorable prognostic sign. A negative test with negative results on examination of a primary inflammatory lesion indicates a non-mycotic condition. They also used oïdiumycin and obtained specific results in patients infected with *Monilia albicans*. Cross tests of the two groups (trichophytosis and moniliasis cases) showed specific reactions with the two extracts.

The nails may be invaded by the *Trichophyta*, *Epidermophyta*, or *Monilia*.

INFECTIONS DUE TO YEAST-LIKE FUNGI

For clinico-pathological purposes we may group together all the organisms which show budding, either in the tissues or on culture media, under this very unscientific term; recognizing that before reporting any such infection as a finished study the organism must be definitely classified botanically.

Avoid the easily mistaken artifacts seen in older sodium hydroxide preparations of skin, exudate or pus which resemble budding forms and even appear to grow hyphæ at times. Check all suspicious material by moist smears without hydrate, stained smears, cultures and animal inoculation.

This group includes the *Monilia*, *Geotrichum*, *Endomyces*, *Saccharomyces*, *Cryptococcus* (*Torula*), *Coccidioides* (not yeast-like), and other less frequently seen genera. Remember that this grouping is based on one morphological trait only and may not be correct botanically.

These organisms may be recovered from the various materials above mentioned or from scrapings of ulcers and mucous surfaces, sputum, or sinus tracts. Cultures, as well as smear preparations, should always be made. Incubate the cultures both at 37° C. and at 22° C., because the rate and profusion of growth varies greatly at these temperatures in different species. The more highly parasitic fungi may not grow at the lower temperature and the more vegetative forms may develop only at a lower degree. The same organism may vary greatly in its type of growth at the different temperatures and in different media. Some forms are typically yeast-like in the presence of protein and definitely filamentous in its absence. Fructification frequently occurs only on aerial hyphæ and some species only produce spores on solid media.

From a case clinically resembling thrush, if definite yeast-like forms are seen, cultures on Sabouraud's isolation medium and on cornmeal agar

should yield typical growth in twenty-four to forty-eight hours. These may be considered *Monilia*. (See references to other works on *Monilia* in these chapters.)

From the buccal mucous membranes and from stools of cases clinically resembling sprue, cultures may yield *Monilia* in almost pure culture and resembling sprue, cultures may yield *Monilia* in almost pure culture and Small pearly colonies

and rather glistening, with a light, fuzzy them, are to be suspected. Study of the surface, yield only yeast-like forms, but if a bit of the medium beneath the colony, including the "fuzz," be studied, the budding hyphæ may be seen. Streaked cornmeal agar plates may be studied under the low power and if covered with a sterile cover slip, the high-dry objective can be used.

Benham¹ stresses these three features as of value in identifying cultures of *Monilia albicans*: (1) Well-developed mycelium showing little tendency to break up into individual elements (2) clusters of spores, chiefly about the hyphæ. (3) hyaline, ellipsoidal, smooth-walled, 2-4 µm. diam. hyaline, ellipsoidal, smooth-walled, 2-4 µm. diam. hyaline, ellipsoidal, smooth-walled, 2-4 µm. diam. *Monilia albicans*

from thrush ferments maltose with the formation of gas, but none appears in saccharose, agreeing in this respect with *Monilia psilosis* from sprue; *Monilia parapsilosis* does not ferment maltose, *Monilia candida* forms gas in saccharose; *Monilia krusei* does not ferment saccharose or galactose and in structure is distinguished from *parapsilosis*. The use of Martin's² technic may be resorted to if extensive work with these strains is performed.

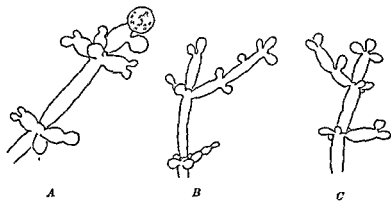


FIG. 35.—A, *Monilia albicans* (or *psilosis*); B, *M. parapsilosis*; C, *M. krusei*.

Stone and Garrod¹⁰ reduced Castellani's more than 42 species of *Monilia* to 8 strains by serologic methods. They used a precipitin reaction, the serum for which was made by injecting rabbits intravenously at five-day intervals for 5 doses rising from the "opacity equivalent of 500 million to 10,000 millions" and bleeding ten days after the last dose. Their extract was made from an emulsified Sabouraud agar slant growth in 10 cc. of saline autoclaved at 15 pounds for thirty minutes and centrifugalized. Mixtures of the serum and homologous antigen gave heavy reactions up to 1:15 dilution and finer flocculation up to 1:25 dilution. They describe their complement fixation technic in other articles to which reference should be made for details. They found a saline washing as effective and yielding sharper details than the suspension originally used as an antigen.

In the skin *Monilia* also show hyphal forms but budding is usually seen along the hyphæ or at their ends. Similar forms are obtained from the mucous surfaces. In pus, exudates or sputum, the yeast-like forms usually predominate. Stains, preferably Wright or Giemsa, may be used to study details. The organisms are Gram-positive and not acid-fast.

If hyphæ are not found, but only budding forms, both in culture and smears, the sugar reactions will usually classify the fungus as one of the *Saccharomycetes* or *Cryptococci*. In old cultures asci may be found.

If budding forms are absent, arthrospores present, the mycelial threads readily disjointed into rather square-ended fragments and certain sugar reactions found, then the fungus is probably an *Oidium* (or *Oöspora*). These organisms may cause acid fermentation but no gas in sugar media. The frequently encountered *Geotrichum asteroides* gives a characteristic granular radiating colony.

Endomyces cannot, ordinarily, be differentiated from *Monilia* in young cultures; the presence of asci in old cultures is the differential feature.

Organisms of the genus *Cryptococcus* (*Torula*) may cause localized abscesses but are most frequently found invading the central nervous system. Cerebrospinal fluid, centrifugalized and stained for differential count, should disclose them. When this condition is suspected, culture the sediment on Sabouraud's medium, incubate at both room and incubator temperatures, and observe for three to four weeks. The organism grows by budding, forms no hyphæ and does not ferment sugars. In tissues it may resemble a lymphocyte but is usually surrounded by a halo and budding may be seen. In smears they may also resemble lymphocytes but have a definite cell wall with an affinity for basic dyes.

Double contoured granular organisms showing budding and growing only by budding, with no asci, are considered as belonging to the genus *Cryptococcus*.

Benham,² studying cultures reported as *Cryptococcus* and *Torula*, concludes that these to

The presence of ... s otherwise resembling *Cryptococcus* but with no buds, suffices to tentatively classify the organisms as *Coccidioides*. These may be found either in fresh material or histologic sections.

The *Histoplasma capsulatum* of Darling is classed as a *Cryptococcus* by Castellani but the work of DeMonbreun⁶ appears to remove it from this group. Thom, of the U. S. Department of Agriculture, considers it related to the genus *Endomyces*. Multiple small spores are seen in endothelial cells on direct microscopic examination. Growth is by budding in some media and at certain temperatures, but definite hyphæ are formed under other circumstances. Large chlamydospores become studded by protrusions of the capsule and again produce hyphæ under favorable conditions. These features would seem definitely to remove this fungus from the *Cryptococcaceæ*. DeMonbreun⁷ has also reported natural infection of the ... ! ...-ial infection by feeding the ... a reservoir from which man ... found in the mesenteric nodes, liver, spleen, peritoneum, gastro-intestinal tract, lungs, heart, meninges, and bone-marrow.

The term blastomyces was used very loosely in the older literature to

describe a number of unrelated yeast-like fungi such as *Monilia*, *Cryptococcus* (*Torula*), and *Geotrichum*. At the present time all this confusion

in all parts of North America and in scattered foci throughout the world. The infection has its highest incidence in males between the ages of twenty and forty. Blastomycosis may be confined to the skin or it may invade the internal organs and produce a severe disease with a mortality of 95 per cent. The skin lesions are chronic and may consist of large verrucous lesions or granulomatous ulcers. Double contoured budding organisms can be found in the pus expressed from the miliary abscesses which occur in the advancing edge of the lesion, or in biopsy sections. The generalized disease frequently involves the lungs and resembles tuberculosis. The characteristic organism may be found in the sputum or in pus aspirated from subcutaneous abscesses. Incubator temperature and type of growth. Cultures grow as a white downy mold with a complete absence of budding forms. The mold-like form can be changed back into the budding form by subculture on blood agar at incubator temperature.

In chromomycosis or chromoblastomycosis which has been studied elsewhere, the fungus appears as a large thick-walled cell with a dark brown pigment in its capsule. Cultures grow as a black mold-like growth with certain fruiting structures which are characteristic of the genera *Hormodendrum* or *Phialophora*. The disease is confined to the skin of the extremities, especially the legs, and is never fatal.

Paracoccidioidomycosis is a highly fatal disease, apparently confined to South America. Some of its clinical features resemble blastomycosis and some coccidioidal granuloma. The causative agent is *Paracoccidioides brasiliensis* (Conant⁴).

INFECTIONS DUE TO OTHER RELATIVELY COMMON FUNGI

I. *Sporotrichosis*.—Cutaneous, subcutaneous, lymphangitic, lymph node, mucosal and deeper infections, may all be caused by fungi of the genus *Sporotrichum*. Cultures on several media, incubated at both 22° C. and 37° C., should be observed for three weeks. The growth is usually seen in five to ten days; the colonies become dark and wrinkled, penetrate the medium and are tough. They can be readily examined through the wall of the test tube with low magnification. Small portions of culture with the underlying medium should be placed on a slide; a drop of alcohol added, some lactophenol and, finally, a cover slip. Examine under the microscope before pressing the slip down and then after flattening the mass by gentle pressure. The alcohol rid the preparation of air bubbles and supports the spores; the lactophenol clears and kills the fungus. The characteristic picture is one of intertwining septate hyphae of rather fine

filamentous form, bearing oval or pear-shaped spores, some attached to the hyphæ directly, but others formed on small, thin conidiophores with the spores grouped in little bunches at the end of the stalk. The spores, when detached, germinate by sprouting hyphæ. Chlamydo-spores may be seen in old cultures. Culture is by far the most certain means of establishing the diagnosis. Spores are rare in smears and in sections of invaded tissue.

II. Aspergillosis.—Pulmonary and auditory canal infections are the chief sources of aspergilli which may be considered pathogens. Skin infections are too easily contaminated by these omnipresent fungi to be considered as due to them, unless after careful cleansing and repeated cultures the fungus can be consistently recovered. In smears and tissues only hyphæ or a few round or oval bodies may be seen.

In cultures growth appears rather rapidly (two to four days), at first white and fluffy but soon developing darker points of color as the fruiting heads appear. Later the entire growth may become black, green, yellow or yellowish-green, depending upon the genus isolated. The colors of the more common ones are: *A. niger*, black or purplish brown; *A. fumigatus*, at first green, then gray; *A. glaucus*, green; *A. candidus*, white or creamy; *A. flavus*, yellowish-green; *A. ochraceus*, yellow.

These fungi may be identified by their characteristic fructifications. A specialized cell of the hypha seems heavier in outline, gives off a branch near the middle of one side, this expands peripherally into a vesicle and on the vesicle numerous chains of spores appear. These chains of spores are borne on short stalks, or sterigmata, or a primary sterigma may support several secondary sterigmata and these bear the spore chains. In some species the spore chains radiate in all directions about a ball-like vesicle, in others they lie close together in a dense brush-like mass arising from an oval vesicle.

III. Penicilliosis.—Fungi of this genus (*Penicillium*) rarely are pathogenic but are often found as contaminants. Mucous membranes, nails, beard and even the lungs, have been reported as infected by these fungi. Repeated positive cultures, after taking all precautions to avoid contaminants, are necessary to establish the pathogenic rôle of this organism.

Numerous species have been studied and are identified by variations in color, form and cultural characteristics. The general cultural characteristics are all that is necessary to place a fungus in this group. The hyphæ are septate and branching, and the terminal branches bear abstricted chains of conidiophores.

IV. Actinomycosis.—The organisms causing this disease are variously called actinomyces, streptothrix, nocardia, trichomycetes, discomycetes, actinobacillus, cohnistreptothrix, actinocladothrix, etc. We shall include all of these organisms, intimately related if not identical, in the general and well-known term "Actinomyces," for the sake of brevity and clarity. These organisms are the "missing link" between the Schizomycetes and Eumycetes, being classed as bacteria by some authorities but included in the fungi by others. Some authors refer to them as the "higher bacteria." (See also Index for further data. These are classified under "Bacteria" by Bergey³ and the Committee on Nomenclature of the American Society of Bacteriologists.)

Their characteristics are the tangled mass of bacilliform mycelia composed of threads less than 1 μ in diameter; thallospore-like formations

which at times resemble chains of streptococci, yet may be definitely the hyphal ends under some circumstances. In culture some grow slowly, and then only in anaerobic cultures. 1 others 37° C.

Pathogens of this group have been grown in infusion broth with 0.1 per cent agar, the growth appearing about 1 cm below the surface as small, white, fuzzy or downy balls which gradually send fine threads further into the medium. Pigment is generally produced and varies with the strain or genus studied. Sporulation occurs by division or fragmentation of the distal ends of the hyphae and, though not always seen in cultures of pathogens, the coils at the distal ends of the hyphae are characteristic. These are seen in cultures on solid media, generally after several subcultures and in the older preparations. Some authorities now claim that only the slowly growing anaerobes are pathogenic. We do not consider this point definitely settled.

found in curettings or material removed by fairly forceful irrigation. In stained tissues the masses are unmistakable.

Mycetoma or Madura Foot is usually associated with actinomyces. The closely related organisms known as *madurella*, *indiella*, *glenospora* and *scedosporium*, may well be included here to convey the warning that multiple sinuses or granulomata, especially of the feet, should be studied with these organisms in mind. The usual smears and cultures should be made in an effort to demonstrate fungus infection. The term paramycetoma is used to designate an infection in which threads but no granules are demonstrable; pseudomycetoma designates the clinical picture without demonstrable fungi.

CONTAMINANTS

In cultures made to recover pathogenic fungi many forms will be encountered which cannot be readily classified. These are the cause of more difficulty than the better known pathogens. Retain the cultures, attempt to repeat their recovery from the same lesion after taking all precautions to avoid contamination and, if successful, then proceed to classify the fungus. Attempts at repeated isolation may not be successful but, in any event, if the organism recovered is strongly suspected of being the etiological agent, final classification should be made. Animal inoculation is advisable in these cases to prove or disprove pathogenicity. After using all known methods and referring to standard texts for assistance, if the fungus cannot be classified, it should be submitted to others more experienced in this field.

A rather common and easily identified contaminant is *Alternaria*, which has coarse hyphae and pyriform conidia, these latter bearing cross and longitudinal striations. These multicellular conidia at times appear in chains. When seen in urine specimens they are often mistaken for para-

sites; their presence in such specimens is due to improperly cleansed receptacles.

Some fungi will be found which do not fructify and merely form masses of white or dark mycelia. Some, or perhaps most, of these, are contaminants. Check such results by repeated cultures and try different media at both room and incubator temperatures. Multiple cultures, varied media, more than one temperature and prolonged observation are essentials to all these studies.

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CHAPTER XXIII

COCCIDIOIDOMYCOSIS

BY STANHOPE BAYNE-JONES

THE term *coccidioidomycosis* was introduced by Dickson about 1938 to cover all types of disease caused by the fungus *Coccidioides immitis*. These types of infection may range from the inapparent, through mild illnesses resembling influenza and "rheumatism," with erythema nodosum, to fatal granulomatous lesions of the skin, lungs and other organs.

I. **Types of Infection.**—Slightly modifying a summary by Smith, these types may be classified as follows:

1. **Initial or Primary Infection** (self-limited, nearly always respiratory and localized in the lungs).

(a) *Inapparent or Asymptomatic Form*, the usual type of infection which involves residents of endemic areas.

(b) *Acute Respiratory*, "Influenzal," or "Pneumonic" Form.

(c) *Ei*iated with erythema
and arthralgia, allergic
joint or muscle pain;
known as "San Joaquin fever," "Valley fever," "desert fever,"
or "desert rheumatism."

(d) *Pulmonary Cavity Form*, probably a complication of the acute respiratory type. The cavity usually closes spontaneously but sometimes persists, although the infection remains well localized.

.ften presenting granulomatous
known as coccidioidal granuloma,
"San Joaquin Valley disease,"

According to Emmons cases of these types may be caused by the closely-related fungus *Haplosporangium parvum*.

II. **Epidemiology.**—Coccidioidomycosis, important both for civilian and military medicine, is known to occur in many parts of the world. The chief endemic foci are in the southwestern United States, notably in the San Joaquin Valley region of California, southern Nevada, Arizona and New Mexico, and the western half of Texas. It is probable that when other arid regions, including North Africa, are investigated as intensively as those listed above, other important areas of endemicity of coccidioidomycosis will be found.

The infection is acquired usually by inhalation of dust containing chlamydospores of *Coccidioides immitis*. While direct transmission from person to person is possible, this is rare. The only significant portal of entry is the respiratory tract. The fungus has been recovered from soil and dust, but where it multiplies outside the lesions of animals is not known. Emmons has suggested that the fungus has a rodent reservoir. The disease shows a definite seasonal trend of incidence, new infections being common during dry and dusty seasons and almost disappearing in rainy seasons.

III. Laboratory Diagnosis.—Clinical diagnosis requires confirmation by laboratory methods. These consist in (1) demonstration of the fungus in materials from lesions, (2) skin tests, and (3) serological reactions, precipitin and complement-fixation tests. Determinations of the sedimentation rate give useful information as to the state of activity of the disease in a patient.

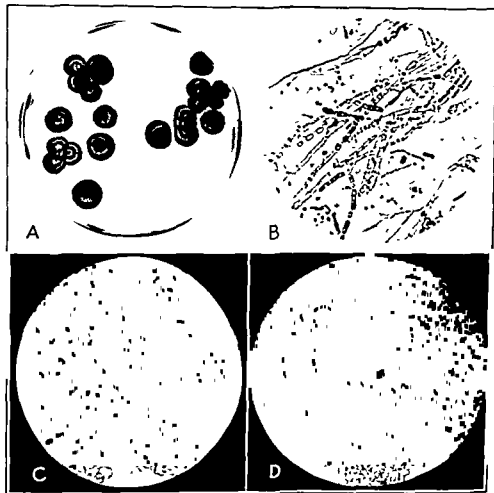


FIG 36.—A, Sputum culture of *C. immitis* on Sabouraud's medium, showing white cottony fungus growth. B, Microscopic appearance of old culture of *C. immitis* showing fragmented chlamydospores. This is the infective form of the fungus occurring in Nature. C, Protoplasm divides into endospores. (Dickson and Gifford, courtesy of Arch. Int. Med.) D, Spherules in cover slip preparation. This shows a double contoured spherule without protoplasm, one with undifferentiated protoplasm and a mature spherule with characteristic endospores. (Photographs, courtesy of Dr. Charles E. Smith.)

1. Demonstration of the Fungus in Materials from Lesions.—(a) *In Sections and Scrapings.*—The form of *Coccidioides* found in sections of tissues and

vary from 10 to 50 μ in diameter.

(b) *By Culture.*

on a differential in Sabouraud's medium or ammonium chloride, 1 per cent sodium acetate, 0.8 per cent tribasic potassium phosphate and 2 per cent agar. This is sterilized in the autoclave at 15 pounds pressure for fifteen minutes. Just before plates are poured 0.04 per cent copper sulfate is added to the melted agar. *Coccidioides* grows scantily on this medium, but very few bacteria or other fungi will grow on it.

On this medium, and more abundantly on Sabouraud's medium the colonies, after incubation at 37° C. or at room temperature, appear in two to seven days as raised discs, penetrating the medium. They develop a leathery surface, which becomes covered with a white layer of aerial hyphae. The colonies become brownish with age.

In culture the spherical bodies put out filaments 2 to 3 μ in diameter. These filaments are branched and septate. Highly refractile arthrospores develop within the mycelial filaments and characteristic spherical chlamydospores are formed at the ends of the hyphae.

2. Skin Tests and Serological Reactions.—Antigens for use in precipitin tests and complement-fixation reactions are made by extracting cultures or from broth in which cultures have been grown.

The material designated coccidioidin is used for diagnostic skin tests, as well as for precipitin and complement-fixation reactions. Coccidioidin skin tests have been used extensively in epidemiological surveys and for diagnosis of all types of infection. It is particularly valuable in the diagnosis of inapparent infections and infections of the milder types resembling "influenza" and associated with erythema nodosum.

According to Smith's method coccidioidin for skin tests is prepared as follows:

specimens of the filtrate of this culture are made on known reactors, and standardization checked on normal and previously infected individuals. Merthiolate is added to a final concentration of 1 to 10,000. The routine test dose is 0.1 cc. of a 1 to 100 dilution of the filtrate injected intradermally in the skin of the flexor surface of the forearm. A positive reaction is a red and swollen area measuring 1 cm. or more in diameter, reaching its peak in thirty-six hours. The test is read as is the Mantoux test. Occasionally, in hypersensitive patients with erythema nodosum, severe local reactions will occur with this dosage, and occasionally in such patients, injections of 0.1 cc. of 1 to 1000 or 1 to 10,000 dilutions may be preferable. However, flaring-up of infection, dissemination of infection, or reactivation of an arrested process has not been seen to follow skin reactions. Coccidioidin has not yet been purified. In the hands of trained observers the use of the material for skin tests has given information of great value both to the Army and to civilian health agencies.

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PART IV

Bacteriology

CHAPTER XXIV

GENERAL TECHNIC

By HARVEY R. LIVESAY

PREPARATION OF MATERIALS

Glassware

I. Cleaning.—The cleanliness and appearance of glassware depends upon the hardness of water in which it is washed. For hard waters, a softener should be used. For milk utensils, trisodium phosphate may be useful. Washing machines or hand washing may be used. Abrasive or alkali soaps should be avoided. New glassware, provided it is of good quality, can be used after simple cleansing with hot water and soap. Boil the glass utensils in soapy water. After testing with the finger to see if all dirt will rinse away, remove the glass and cool, so it can be handled without danger of breaking. Rinse thoroughly in running warm water to remove all soapy material; re-rinse in distilled water and place on a clean drainboard to dry.

For used glassware, more elaborate cleansing methods are required. Contaminated pipets, immediately after use, are placed for thirty minutes or longer in tall jars or cylinders containing 2 to 5 per cent compound cresol solution. To wash them attach a large rubber tube to the cold water faucet and insert the ends of several pipets into the hose; turn on the water and force it through until they are clean; then remove, rinse in distilled water and stand them on end to drain and dry. A suction pump or an automatic pipet washer may be used. If the pipets are not clean they should be soaked in dichromate-sulfuric acid cleaning solution (see page 355) and then washed.

Test tubes, Petri plates, and flasks containing cultures of pathogenic bacteria should be autoclaved, and their contents emptied into the garbage can. They are then boiled in soapy water, cleansed with a brush, rinsed in tap water, drained, dried and polished, if necessary. Test tubes smeared

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cleaning solution either in large glass jars or earthenware containers for twenty-four hours, then removed with tongs and rinsed thoroughly in running water, after which it is drained and dried.

Used glass slides and cover slips are cleaned by boiling in soap and water, and rinsing in tap water. They are then placed in a porcelain dish,

covered with dichromate-sulfuric acid cleaning solution and soaked overnight. Pour off the cleaning solution and rinse in tap water; then wash in 70 per cent alcohol, dry and polish. New slides and cover slips should not be boiled in soap and water, but placed in the cleansing solution and then washed in tap water and later in alcohol. Syringes and needles are thoroughly cleaned in tap water, distilled water, 70 per cent alcohol, then with ether and dried at room temperature.

II. Plugging, Wrapping and Storage.—After the glassware has been cleaned and dried it is inspected, and any pieces that are not clear and clean are removed for recleaning. Test tubes and flasks for culture work are plugged with cotton. Tubes for agglutination and other work are inverted in clean wire baskets and sterilized. For large flasks, etc., the cotton plugs may be wrapped in cheesecloth; and as an added protection after sterilization, they may be covered with caps of heavy paper, muslin, lead foil, or parafilm. Syringes are dismantled and the parts wrapped in heavy unbleached muslin, which is pinned together and labeled. Syringes may be dismantled and placed in large cotton plugged test tubes. Petri plates are placed in round or square metal boxes for sterilization or they may be wrapped individually with paper. Pipets are wrapped individually with paper, or can be placed in copper boxes for sterilization. After sterilization all glassware is labeled and stored in a clean cabinet protected from dust.

STERILIZATION

Satisfactory sterilization may be secured by dry heat, steam under pressure, steam not under pressure, chemicals or by filtration.

I. Dry Heat.—Dry heat, in an electric or gas oven registering from 170° to 190° C. for one hour is sufficient provided the containers are not large. If sterilizers are well filled, leave air space between containers. Large containers of vaccine bottles may require four to five hours' time. Cotton plugs and paper wrappings are destroyed at temperatures higher than 190° C. Place the glassware in cans or containers, the tops of which are loosened, on the bottom shelf of the oven; and place all wrapped or plugged materials on the top shelf. Start the heat, load the sterilizer and watch the temperature. When the heat reaches 170° C., regulate it so that it will remain at that point, and set a time clock for the desired period of sterilization.

II. Steam Under Pressure.—An autoclave, with or without a jacket, is preferred for the sterilization of linen, cotton goods, rubber, glassware and culture media which are not injured by high temperatures. When sterilizing bulky materials the time and pressure must be adjusted so as to secure the desired penetration. For sterilization with a jacketed autoclave, use the following procedure: Turn the steam into the jacket, with the exhaust open, and allow the heat to rise slowly. Close the exhaust to the jacket, load the sterilizer and close the door. Open the exhaust to the inside chamber, turn the steam into the chamber and when the air has been forced out through the exhaust, close it. Allow the pressure to rise to 15 pounds or more as desired. Set the time clock for the desired length of sterilization. Ordinarily 15 pounds' pressure or 120° C. at steam outlet for fifteen to thirty minutes will suffice. Cut off the steam and allow the pressure to fall; or if a vacuum is attached, cut off the steam and

connect the vacuum in order to dry the contents. Allow the pressure to fall. If the autoclave has no jacket, the preliminary heating is not required. Care should be used to avoid lowering the temperature too rapidly as a vacuum may be produced which may pull the solutions and plugs out of the containers.

The autoclave can be used as an inspissator for Loeffler's and Petroff's media, provided the inside temperature is kept below 100°C . Warm the jacket and then place the media in the autoclave with the desired slant. Close the door and turn the steam into the chamber. Open the exhaust to the inside chamber and with the first appearance of steam at the exhaust, close it. Allow the pressure to rise to 15 pounds and cut off the steam. Let the pressure fall of its own accord. Repeat on two succeeding days. The above procedure is satisfactory for Petroff's and other egg media. For Loeffler's blood serum, the pressure should be held at 10 pounds for fifteen minutes, and then cut off and allowed to cool. This is repeated on three succeeding days.

III. Steam Not Under Pressure.—The Arnold sterilizer is used for materials that may be damaged by overheating. Boil the water in the pan to insure plenty of live steam, remove the top or open the door and place the material in the sterilizer. Heat for the desired time and repeat the procedure on the two succeeding days.

IV. Chemicals.—Compound cresol solution in strengths of from 2 to 5 per cent is commonly employed to sterilize discarded glassware or old cultures, and for cleansing floors, tables, rubber gloves, etc. Rubber stoppers are conveniently sterilized by boiling in 5 per cent phenol solution, for at least twenty minutes.

V. Filtration.—Sterile Berkefeld, Mandler, Seitz or other filters are employed for removing bacteria from liquids that may be damaged or destroyed by heat, such as antisera, dextrose solutions, special liquid media, etc.

STAINS AND OTHER SOLUTIONS

Saturated alcoholic solutions of the basic aniline dyes are kept in stock and used for the preparation of the more dilute solutions as desired. The solubilities of various dyes in water and in 95 per cent alcohol are indicated

..... The stains commonly employed for

1. **Methylene Blue.**—Aqueous solution prepared by adding 95 cc. of distilled water to 5 cc. of saturated alcoholic solution of methylene blue.
 2. **Loeffler's Alkaline Methylene Blue.**—Made by adding 70 cc. of 1 to 10,000 solution of KOH (0.1 cc. of 10 per cent KOH in 100 cc. distilled water) to 30 cc. of saturated alcoholic solution of methylene blue.
 3. **Safranin.**—To 10 cc. of the saturated alcoholic solution add 90 cc. of distilled water.
 4. **Bismarck Brown.**—Dissolve 0.2 to 1 per cent of the powder in boiling water, cool and filter.
 5. **Carbolfuchsin (dilute).**—To 10 cc. of carbolfuchsin described under the Ziehl-Neelsen stain add 90 cc. of water.
- For ordinary use these stains are applied for two to five minutes, after which the slide is washed with water, blotted and dried.

TABLE 53.—SOLUBILITY OF DYES (PREPARED BY CONN)*

Dye	Water	Alcohol, 95%
Methylene blue (chloride)	3 55	1.48
Fuchsin (basic) Rosaniline (chloride)	0.39	8 16
Para Rosaniline (chloride)	0 26	5 93
Para Rosaniline (acetate)	4.15	13 63
New fuchsin (chloride)	1.13	3.20
Methyl violet	2.93	15 21†
Crystal violet (chloride)	1 68	13 87
Eosin Y (Na salt)	44 20	2 18
Eosin, ethyl	0 03	1 13
Eosin B (Na salt)	39 11	0.75
Erythrosin (Na salt)	11.10	1 87
Bismarck brown y	1.36	1 08
Picric acid	1 18	8 96
Pyronin B (iodide)	0 07	1 08
Pyronin Y	8.96	0 60
Safranin	5 45	3 41
Toluidine blue (O)	3 82	0 57
Thionin	0 25	0 25

II. Differential Stains.—A. Gram's Method.—This is the most important of all bacteriological stains. There are many modifications of the original method in use, but the basic principle has not changed; namely, the use of a good primary violet stain, followed by Gram's iodine, a decolorizing agent, and a contrast counterstain.

1. Reagents.—(a) For the *primary stain*: Crystal violet, saturated alcoholic solution, 1 part; ammonium oxalate, 1 per cent aqueous, 4 parts.

(b) *Gram's Iodine*.—Iodine, 1 gm.; potassium iodide, 2 gm.; distilled water, 300 cc.; or distilled water, 240, dissolve and add 60 cc. of 5 per cent aqueous sodium bicarbonate.

(c) *Decolorizer*.—Ninety-five per cent alcohol or acetone.

(d) *Counterstain*.—Aqueous, simple contrast stains, of safranin, Bismarck brown, or dilute carbolfuchsin.

2. *Technic*.—Prepare even smears, dry in air and fix by heating over flame. Apply primary stain for one to two minutes. Pour off excess and add Gram's iodine solution for one minute. Wash in water and apply decolorizer until no further traces of violet can be detected. Wash in water and apply counterstain for one minute. Wash in water, blot and air dry.

3. *Results*.—All newly prepared stains should be checked with known Gram-positive and Gram-negative organisms, as staphylococcus and *E. coli*, before use. Gram-positive organisms retain the violet color; while the Gram-negative ones lose the violet and are colored by the counterstain.

B. *Neisser's Method for C. Diphtheriae*.†—This method depends on the demonstration of granules. Many lots of methylene blue contain an excess of alcohol-insoluble foreign material, and this accounts for the irregularity of the staining quality if the dry dye is weighed and used to prepare the staining solution

1. *Reagents*.—(a) *Neisser No. 1*.—For a satisfactory stain, mix 1 cc. of a saturated 95 per cent alcoholic solution of methylene blue with 50 cc.

of a freshly prepared 5 per cent solution of glacial acetic acid in distilled water.

(b) *Neisser No. 2 (Counterstain)*.—Aqueous Bismarck brown, or safranin.

2. *Technic*.—Prepare films, air dry and fix by heat. Apply *Neisser No. 1* stain for one minute. Wash gently in tap water. Apply counterstain for one minute. Wash, blot and dry.

C. *Ziehl-Neelsen Method for Genus Mycobacterium*.—This method is used for separating acid-fast from non-acid-fast organisms and depends upon a primary stain, a decolorizer and a contrast counterstain.

1. *Reagents*.—(a) For the *primary stain*, mix 10 cc. of a saturated alcoholic solution of fuchsin (basic) with 90 cc. of a 5 per cent solution of phenol in distilled water.

(b) *The decolorizer* is usually a dilute acid or acid-alcohol. Three per cent hydrochloric acid in 95 per cent ethyl alcohol is satisfactory for routine work.

(c) *Counterstain*.—Loeffler's alkaline methylene blue.

2. *Technic*.—Prepare films, air dry and fix by heat. Apply carbolfuchsin and heat gently until steam appears over surface. Allow to steam for five minutes. Wash in water and decolorize with acid-alcohol until a faint pink remains. Wash in water and counterstain with Loeffler's alkaline methylene blue for one minute. Wash in water and air dry.

3. *Results*.—Acid-fast organisms are stained red. Non-acid-fast organisms are stained blue.

D. *Hiss' Method for Capsules*.—1. *Reagents*.—(a) *The staining solution* is prepared by mixing 10 cc. of saturated alcoholic solution of gentian violet or fuchsin with 90 cc. of distilled water. Some prefer undiluted Gram's crystal violet or carbolfuchsin solutions.

(b) *Copper sulfate*, 20 per cent aqueous solution.

2. *Technic*.—Films are prepared from fresh material or by mixing cultures of organisms with animal serum, preferably beef serum. Dry in air but do not heat. Apply staining solution; heat until steam appears and allow to stain for one to two minutes. Wash off the stain with the copper sulfate solution. Blot dry (do not wash) or examine wet under a cover slip.

3. *Results*.—The capsule should show a lighter color than the body of the organism.

E. *Churchman's Capsule Stain*.—Prepare films and air dry. Flood the film with Wright's stain and allow to remain until it is almost evaporated to dryness. A pinkish color replaces the original blue of the stain. This takes from three to five minutes. Wash off rapidly with water or with Clark and Lub's buffer pH 6.4 to 6.5. Dry with a fan but do not blot. The body of the organism stains blue; the capsular substance a purplish-pink which is often surrounded by a deeper purplish-pink peripheral zone or capsular membrane.

(b) *Method*.—The specially cleaned.

ernight, pour off

r. Pour off water,

cover with 95 per cent alcohol, and add about 15 drops of concentrated ammonium hydroxide. A few minutes later pick out one at a time with forceps, and dry carefully with lens paper. (Never touch with fingers.) Place in a Petri dish cleaned in the same manner. Heat in a hot air oven

at 275° to 300° C. for one hour. If a drop of tap water is placed on the slide or cover slip and it remains in place and spreads out evenly about its edge, the slides are properly cleaned.

1. **Reagents.**—A special reagent is necessary for fixation, instead of heat. It is composed of two solutions, as follows:

(a) **Solution A.**—Ferric chloride (1 to 20 aqueous solution), 1 part; tannic acid saturated aqueous solution, 3 parts; this solution improves with age, and should be at least a week or two old. It should be kept in stock and filtered before use.

(b) **Solution B.**—Aniline, 1 part; 95 per cent alcohol, 4 parts.

(c) **Staining Solution.**—For staining, after using the fixing solution, mix 30 cc. of Loeffler's methylene blue with 3 cc. of solution B. This is ready for use and keeps well. If a red stain is desired in place of the blue, use carbolfuchsin.

2. **Technic.**—After careful preparation of cultures in broth to insure a good motile strain, transplant to nutrient agar and incubate from six to twelve hours. Gently remove a loop of the growth from the agar and place it in a tube of sterile tap water. Hold the loop in the tube of tap water without agitation until you see a slight opalescent change in the water, around the loop of growth. Remove loop and place the tube in the incubator for one-half hour to allow diffusion of the organisms. Gently remove tube from incubator and, without shaking, take several small loopfuls of the suspension and place on the specially prepared slides or cover slips. (Do not spread.) Air dry, add 8 drops of solution A, and immediately add 1 drop of solution B. Allow the combination to act at room temperature for two minutes. Wash in tap water and touch the edge of the cover slip or slide to a piece of filter paper. (Do not blot.) Flood the slide or cover slip with the staining solution, either the special methylene blue or carbolfuchsin, and allow to act at room temperature for two to three minutes. Wash thoroughly in tap water, air dry and mount in balsam.

G. Spore Stain.—Prepare a film in the usual way and fix with heat. Stain with carbolfuchsin as in the Ziehl-Neelsen method. Wash in hot tap water. Rinse rapidly with 95 per cent alcohol. Apply Loeffler's methylene blue for two to five minutes. If the film is thick, pour off methylene blue and add more blue. Rinse in tap water, blot and dry.

H. Fontana's Treponema Stain.—Fontana's method is particularly adapted for the staining of *T. pallidum* in smears from chancres. Three solutions are required:

1. **Reagents.**—

(a) **Fixing**

Acetic acid (glacial)	1.00 cc.
Formalin (40 per cent)	2.00 cc.
Distilled water	100.00 cc.

(b) **Mordant**

Phenol (liq. crystals)	1.00 cc.
Tannic acid	5.00 gm.
Distilled water	100.00 cc.

(c) **Silver Solution**

Silver nitrate	0.25 gm.
Distilled water	100.00 cc.

To a part of the silver nitrate solution, add a dilute solution of ammonia, drop by drop, until the precipitate that forms is dissolved. Then add, drop

by drop, a small quantity of the silver nitrate solution until a faint opalescent color is produced.

2. **Technic.**—Spread a thin film of material on slide, air dry, and treat with the fixing solution for one to two minutes. Renew the fixing solution during this time. Wash in running water. Pour on mordant and heat gently until steam rises; continue for one-half minute. Wash in running water and then in distilled water. Rinse with the silver solution, add fresh silver solution and heat until steam rises. Wash, air dry, and mount.

3. **Results.**—*T. pallidum* appears dark brown or black on a light brown background.

III. **Relief Methods.**—India ink or Congo-red (Benians) 2 per cent, may be used as contrast materials in the demonstration of organisms. The India ink (Higgin's) should be homogeneous and free from gross particles or bacteria. Place a drop of the material to be examined on a slide, add a drop of India ink, mix and spread evenly, similar to a blood smear. Air dry and examine with the micro-scope. Benians uses 2 per cent Congo-red in place of the India ink and after drying treats the film with acid-alcohol which converts the stain to a purplish-blue color.

IV. **Miscellaneous Solutions.**—In the bacteriological laboratory, a number of miscellaneous solutions are required for routine use. Some of these are listed below.

1. **Sodium Chloride (Physiological Salt) Solution.**—

Sodium chloride	5.5 gm.
Distilled water	1000.0 cc.

2. **Buffer Solution.**—

Sodium dihydrogen phosphate (NaH_2PO_4)	28.81 gm.
Sodium hydrogen phosphate (Na_2HPO_4)	125.00 gm.
Distilled water	to 1000.00 cc.

3. **Sodium Chloride Solution, Buffered.**—

Buffer solution (above)	20.0 cc.
Sodium chloride	5.5 gm.
Distilled water	to 1000.0 cc.

4. **Sodium Citrate-Sodium Chloride Solutions.**—

	1 per cent	2 per cent	10 per cent
Sodium citrate c.p.	10.0 gm.	20.0 gm.	100.0 gm.
Sodium chloride	5.5 gm.	5.5 gm.	5.5 gm.
Distilled water	1000.0 cc.	1000.0 cc.	1000.0 cc.

5. **Potassium Oxalate Solution.**—

Potassium oxalate	2 gm.
Sodium chloride	5 gm.
Distilled water	100 cc.

Coagulation may be prevented by adding 1 cc. of this solution to 10 cc. of blood.

6. **Sodium Carbonate Solution.**—Two gm. per liter of water may be used for buffering instruments, as it prevents corrosion.

BIOCHEMICAL REAGENTS AND TESTS

1. **Indicators for Media.**—Phenol red in 0.02 per cent aqueous solution or 0.04 per cent aqueous alcohol solution, and 0.1 per cent aqueous bromothymol blue solution are commonly employed. A definite quantity of one of these indicators is incorporated with the bacterial media used.

II. Indol Test.—Bohme's reagents, which consist of two solutions, are satisfactory for this test.

1. Reagents.—

- (a) p-Dimethylaminobenzaldehyde 4 gm.
 Ethyl alcohol, 95 to 96 per cent 380 cc.
 Hydrochloric acid, concentrated 80 cc.
- (b) Potassium persulfate, saturated aqueous solution.

The organism is grown for five days in 1 per cent peptone water. To this culture add 1 cc. of ether, shake and allow to stand. Then allow 1 cc. of the *Solution* (a) to run down the side of the tube. If no color appears in one minute, add 1 cc. of the *Solution* (b). A positive reaction is shown by the appearance of a pale pink to a deep magenta color.

III. Nitrate Reduction, Posvy's Method.—1. Reagents.—

- (a) 1 gm.
 22 cc.
 of dilute
- (b) Sulfanilic acid 0.5 gm.
 Dilute acetic acid 150 cc.

Grow the culture for five days at 37° C. in broth containing 0.1 per cent KNO_3 . Add 1 cc. of the *Solution* (a) and follow this by 1 cc. of the *Solution* (b). A pink, red or maroon color is positive; no coloration indicates a negative reaction.

IV. Ammonia (NH_3).—A peptone water culture, grown for five days at 37° C. is tested by adding 0.5 cc. Nessler's reagent.

Brown color = positive
 Faint yellow color = negative

V. Hydrogen Sulfide (H_2S).—Test organisms by inoculation on lead acetate agar. This medium is prepared by sterilizing extract broth, containing 4 per cent peptone and 2.5 per cent agar, and adding to it an equal quantity of a sterile 0.1 per cent aqueous solution of basic lead acetate.

Brown or black color = positive
 No color = negative

VI. Reductase Test (R).—To a twenty-four-hour broth culture add 1 drop of 1 per cent aqueous methylene blue, and incubate at 37° C. Complete decolorization indicates strong reduction; a green coloration is weakly positive and no decolorization is a negative reaction.

VII. Catalase (C).—Use a twenty-four-hour agar slant culture incubated at 37° C. Pour 1 cc. of H_2O_2 (10 volumes) over the growth, and set the tube in an inclined position. If gas bubbles are produced the reaction is positive, if no gas is produced it is negative.

VIII. Methyl-red Test (M.R.).—Add 5 drops of 0.04 per cent solution of methyl red in 60 per cent alcohol, to a culture grown four days at 37° C. in glucose phosphate medium [proteose peptone (Witte or Difco) 0.5 gm., K_2HPO_4 0.5 gm., glucose 0.5 gm., water 100 cc., pH 7.5]. A red color is reported as positive and a yellow color as negative.

IX. Voges-Proskauer Test (V.P.).—Add 5 cc. of a 10 per cent solution of KOH to a culture grown for four days at 37° C. in glucose phosphate

medium. The color develops slowly, and the test should be read after eighteen to twenty-four hours. A pink fluorescence indicates a positive, and no color a negative reaction.

X. Oxidase Test.—1. Reagents.—

Dimethylparaphenylenediamine hydrochloride*	1 gm.
Distilled water (tap water may be used)	100 cc.

Prepare a solution by adding 1 gm. of the dye component to 100 cc. of distilled water. Store in rubber-stoppered flasks. The solution should be prepared freshly each week.

2. Technic.—(a) Apply a loopful of the solution to the surface of a single likely looking colony. Some workers prefer to flood the entire plate at once by pipeting from 1 to 2 cc. of the oxidase reagent to the surface of the medium.

(b) Colonies of *Neisseria* will show a series of color changes from pink to maroon and finally black.

(c) The organisms are usually dead by the time the black stage is reached but if the colony is subcultured before this stage, viability can be maintained.

CULTURE MEDIA

The microorganisms encountered in medical bacteriology necessitate the use of a variety of culture media. This outline is limited to the technic of preparation and to the description of a few of the common media.††

Dehydrated culture media prepared by reliable manufacturers and supplied in the dry form can be advantageously substituted for the homemade media. Directions for their use are enclosed with each preparation.

Many media are prepared from nutrient broth containing the soluble constituents of meat, which is a canned extract or an infusion of fresh meat combined with peptone. Many peptones are on the market, but for routine use Difco Bacto peptone is recommended, and, unless otherwise noted, is the one employed in the formulæ given here.

1. Adjustment of Reaction.—The hydrogen ion concentration of media may be determined and adjusted either by the electrometric or the colorimetric method.

1. Electrometric Method.—Special apparatus using the glass electrode is employed. For a discussion of the principles involved see Chapter XIV and the references listed there.

2. Colorimetric Method.—This method, which is commonly used in determining the reaction of culture media, is based on the fact that certain substances known as indicators display a range of different colors in solutions of different hydrogen-ion concentration. Of the indicators discussed in the section on chemistry, those most used in media titration include phenol red with a color range from pH 6.8 to 8.4, bromthymol blue with a range from pH 6.0 to 7.6 and cresol red of which the range is pH 7.2 to 8.8.

* OR Tetramethylparaphenylenediamine hydrochloride solution may be made and used in exactly the same way. It is less toxic and gives the colonies a lavender color, which eventually turns purple. It has the disadvantage of sometimes coloring the surrounding medium, and is more expensive than the dimethyl.

† For a comprehensive description of the many available media, see *A Compilation of Culture Media*, by Levine and Schoenlein, Baltimore, Williams & Wilkins Company, 1930.

† Descriptions of the more commonly used media are given in the *American Public Health Association's Manual of Diagnostic Procedures and Reagents*, 1941.

For the titration of media sets of color standards are used. Each set usually consists of 9 color standards containing 10 cc. of buffer solution and 0.5 cc. of indicator and increasing by 0.2 pH in each tube over the range given by the particular indicator, a tube of distilled water, and a comparison tube.

Phenol red and bromthymol blue sets are prepared and distributed from the Army Medical School, Washington, D. C. They may also be secured from the various laboratory supply houses.

The set is used in the following manner for the titration of media. A fuller discussion of colorimetric methods is given in Chapter XIV.

Select the standard tube of the desired pH and place it in the right front hole of a "comparator block," and place immediately behind it a tube of the medium to which no indicator has been added. In the left rear hole place a tube of distilled water and in the left front hole a tube containing 10 cc. of medium to which has been added 0.5 cc. of indicator. Hold the comparator block toward the daylight and determine whether the medium has the same color as the pH standard. If not, add to the medium, measured quantities of 0.1 N NaOH or 0.1 N HCl, as required until the colors are alike. From the volume 0.1 N acid or alkali used to adjust 10 cc. of the medium to the desired pH, the amount required for 1 liter may be estimated by multiplying the number of cc. used by 100. After the final addition of NaOH or HCl, recheck the pH of the medium and make any other adjustments indicated.

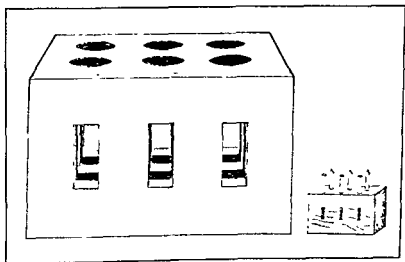


FIG. 37.—Comparator block. For use in the colorimetric titration of culture media.

ing steam as they become more acid after autoclaving. Peptone water is usually rendered more alkaline by sterilization. The addition of agar causes little change in the reaction of a fluid medium, but gelatin increases the acidity.

II. Clarification.—Media may be clarified either by centrifugalizing or by filtration through cotton, gauze, cloth or paper. If necessary, eggs may be added and coagulated by heat just prior to filtration.

III. Distribution.—The media should be placed in sterile glass flasks or tubes before sterilization. As a rule this is done by placing the fluid medium in a large container or funnel from which it is delivered through a hose controlled by a pinch-cock. While the quantities are not usually measured, the following approximate amounts have been found to be convenient for 150 x 16 mm. test tubes; for agar slants 5 cc., agar unslanted 10 to 15 cc. and for fluid media 10 cc.

IV. Storage.—After sterilization, culture media should be stored in a refrigerator or cold room, and the necks of the flasks or tubes should be protected with caps of paper, or lead foil. In certain places, especially in the tropics, fungi commonly grow through unprotected moist cotton stoppers and contaminate the medium inside the container.

V. Formulæ for Basic Culture Media.—

Meat Extract Broth

(For routine use)

1. Beef extract	3 gm.
2. Peptone	10 gm.
3. Sodium chloride	5 gm.
4. Distilled water	1000 cc.

Add the weighed ingredients to the distilled water and heat slowly on a water bath to 65° C., stirring until dissolved. Make up the loss in weight with distilled water, and adjust the reaction so that the final pH will be between 7.2 and 7.4. Boil over a free flame, cool to 25° C., make up the lost weight with distilled water, clarify and if necessary readjust the reaction. Distribute in test tubes or flasks and sterilize in the autoclave at 15 pounds' pressure for fifteen minutes.

Meat Extract Broth

(Standard for water analysis)

1. Beef extract	3 gm.
2. Peptone	5 gm.
3 Distilled water	1000 cc.

Prepare as directed above but adjust the final reaction to between pH 6.4 and 7.

Meat Extract Agar

(For routine use)

To 1000 cc. of meat extract broth add 20 to 30 gm. powdered agar

the reaction to pH 7.2 to 7.4. Tube or place in flasks and autoclave at 15 pounds for fifteen minutes.

Meat Extract Agar
(Standard for water analysis)

To 1000 cc. of meat extract broth for water analysis add 15 gm. of best quality agar. The reaction should be adjusted to pH 6.4 to 7.

Meat Infusion Broth

- | | |
|--|----------|
| 1. Beef or veal round, free from fat, ground | 500 gm. |
| 2. Distilled water | 1000 cc. |

Mix the meat and water and infuse in the icebox for eighteen to twenty-four hours. Heat in the Arnold sterilizer or over a low flame for one hour. Squeeze through cheesecloth until 1000 cc. are obtained. Add 5 gm. of sodium chloride, and 10 gm. of peptone and dissolve over the flame. Filter, and add distilled water to 1000 cc. Titrate to pH 7.4. Tube or flask and autoclave at 15 pounds' pressure for fifteen minutes.

Meat Infusion Agar

Add to 1 liter of meat infusion broth 20 gm. of agar and proceed as in the preparation of meat extract agar. Adjust to pH 7.4.

Gelatin, Nutrient

- | | |
|---------------------------------------|----------|
| 1. Meat extract broth | 1000 cc. |
| 2. Sheet gelatin (purified) | 120 gm. |

Place the broth in a double boiler and add the gelatin. Weigh and dissolve by heating. When gelatin is dissolved, titrate to pH 7.4. Re-weigh and add distilled water to the original weight. Add one egg mixed with a small quantity of water to clarify, re-heat slowly until the egg is firmly coagulated. Filter through cotton. Tube in 10-cc. portions and sterilize in the Arnold for twenty minutes on three successive days.

VI. Formulæ for Special Culture Media.—

Huntoon's Hormone Medium

- | | |
|--|----------|
| 1. Beef heart (fresh, finely ground) | 500 gm. |
| 2. Peptone | 10 gm. |
| 3. NaCl | 5 gm. |
| 4. Egg, whole | 1 |
| 5. Agar (Bacto) | 20 gm. |
| 6. Water, distilled | 1000 cc. |

Place all ingredients in an enamelware vessel and heat over the flame to 68° C., stirring constantly. Titrate to pH 8. Cover the vessel and place in the Arnold sterilizer for one hour. Remove the vessel from the sterilizer and separate the firm clot from the side of the vessel. Return to the Arnold sterilizer for one and one-half hours. Remove and allow to stand in an inclined position, at room temperature, for ten minutes. Carefully decant or pipet off the clear portion and filter it through a fine wire sieve into tall cylinders. Allow to stand for fifteen to twenty minutes and remove the fat from the surface. The medium may be further cleared by filtering through glass wool, asbestos wool or by centrifugalizing. Tube in 10-cc. portions and sterilize in the Arnold for twenty minutes on three successive days.

Glucose Agar

To 1 liter of melted meat extract agar or meat infusion agar, add 10 gm. of glucose and heat slowly until dissolved. Titrate and adjust the reaction to the original pH of the agar. Place in tubes or flasks and sterilize in the Arnold on three successive days.

Blood Agar

Add 5 to 10 per cent of sterile defibrinated blood (preferably horse blood) to meat infusion agar or meat extract agar that has been melted and cooled to 45° C. Pour into plates, or place in tubes and slant; incubate to determine sterility.

Chocolate Blood Agar

Add 5 per cent of sterile defibrinated blood to melted meat infusion agar at 50° to 55° C., mix carefully to avoid bubbles and slowly raise the temperature to 75° C. Pour into plates or tube and slant. Incubate to determine sterility.

Serum Agar

To 1 liter of melted meat infusion agar add 100 cc. of sterile normal horse serum. Place in sterile tubes and slant, or pour into Petri plates. Incubate to determine sterility. Sterile ascitic or hydrocele fluid may be used instead of the serum.

Liver Infusion Agar

(For *Br. abortus*)

1. Beef liver infusion	500 gm.
2. Distilled water	500 gm.
3. Agar	20 gm.
4. Peptone	10 gm.
5. NaCl	5 gm.
6. Egg albumin	10 gm.

To prepare 1, weigh 500 gm. of ground beef liver, add 500 gm. of distilled water and infuse in a cool place for twenty-four hours. Strain through cheesecloth and collect the 500 gm. of infusion. Now add 3 to 2 and autoclave for thirty minutes at 15 pounds' pressure. Dissolve 4 and 5 in 1. Dissolve 6 in 10 cc. water. Combine all ingredients except the egg albumin, and make up the weight lost by evaporation. Adjust reaction to pH 7. Cool to 50° C. and add dissolved egg albumin. Heat to 100° C. for one and one-half hours. Strain through a fine wire sieve and filter through clean glass wool. Readjust to pH 7. Tube in 15-cc. amounts and autoclave at 15 pounds' pressure for thirty minutes. When needed, melt and make slants or pour into plates.

Trypsin Agar

1. Beef or veal round (free from fat, finely ground)	500 gm.
2. Distilled water	1000 cc.

Place 1 and 2 in a container and add 20 per cent NaOH until slightly alkaline to litmus. Cook the ingredients at 75° C. for five minutes, cool to 37° C. and add 0.5 gm. of trypsin (Bacto). Incubate at 37.5° C. for five hours. Remove 5 cc. of the liquid and add 5 cc. of 1 N NaOH and 1 cc

of dilute aqueous CuSO_4 . A pink color indicates that trypsinization is complete. If no reaction is obtained reincubate for one hour, and re-test. When trypsinization is complete, acidify slightly with glacial acetic acid and bring slowly to the boiling-point and hold for fifteen minutes. Filter through wet paper, add 20 gm. agar and 5 gm. NaCl . Heat to dissolve the agar, clear with an egg, adjust the reaction to pH 7.6, and autoclave for fifteen minutes at 15 pounds.

Veal Infusion Brain Broth
(For Streptococci and Anaerobes)

With a large bore pipet or tube place approximately 10 cc. of ground fresh calf brain into the bottom of a large tube (200 x 25 mm.) and add 35 cc. of veal infusion broth pH 7.6. Autoclave at 15 pounds for twenty minutes, cool; remove 10 cc. of the supernatant fluid and check the reaction. If the reaction is pH 7.4 to 7.6 it is satisfactory, but if there has been a drift of the pH, adjust to pH 7.6. Estimate from the titration of the 10 cc. the amount needed for the bulk of broth and correct the reaction. Then fill the tubes with similar quantities of the brain tissue and broth. Sterilize in the autoclave at 15 pounds for twenty minutes. Incubate at 37° C. to determine sterility.

Robertson's Medium
(For Anaerobes)

1. Beef heart	500 gm.
2. Peptone	10 gm.
3. Water, distilled	1000 cc.

Obtain a fresh beef heart, remove all fat, fascia and blood vessels. Chop or grind the muscle into small pieces. Add 1 and 2 to 3 and bring to a boil, adjust the reaction to pH 8. Continue to simmer for one and one-half hours. Readjust the reaction. Separate the broth from the meat and place in flasks. Sterilize in the autoclave at 15 pounds for fifteen minutes. Place the meat on clean filter paper and dry in the oven at 56° C. for forty-eight hours. Place the desired quantity of dried heart in the bottom of a test tube and add to it a 10-cc. amount of the above broth. Autoclave, cool, remove broth and retitrate. Adjust the reaction of the broth to the desired pH to cover the fall in pH attending sterilization. Now fill the tubes with the same quantity of meat and broth and autoclave at 15 pounds for thirty minutes. The final pH should be 7.4 to 7.6.

Calcium Carbonate Broth
(For Pneumococci)

1. Meat infusion broth	1000 cc.
2. Glucose	10 gm.

Add 2 to 1 and dissolve by heating. Adjust the reaction to pH 7.6. Place clean marble chips, CaCO_3 , in the bottom of test tubes and add the broth. Sterilize for fifteen minutes in the Arnold on three successive days.

Blood Culture Medium (Kracke)

1. Heart muscle extract	800 cc.
2. Brain suspension	110 cc
3. Sodium citrate	1 gm.
4. Dextrose (Bacto)	10 gm
5. Proteose peptone (Difco)	10 gm.
6. Disodium phosphate	2 gm.
7. Sodium chloride	4 gm.

Obtain fresh beef hearts and brains. Trim the fat from the hearts and grind the muscle in a small bore meat grinder. Weigh 500 gm. of the heart muscle and mix with 1000 cc. of distilled water. Place in the ice-box overnight. Press through four layers of cloth gauze. Now heat the filtrate to the boiling-point, and filter through a small mesh wire or copper gauze.

Macerate or grind the beef brain, weigh 250 gm. and mix with 500 cc of distilled water and infuse overnight in the refrigerator. Filter through four layers of cloth gauze. Slowly heat the filtrate to the boiling-point with constant stirring. Do not filter.

Mix 1, 2, 3, 4, 5, 6 and 7, heat until all ingredients are in solution. Adjust the reaction to pH 7.4. Place in tubes or flasks in 50-cc. portions and sterilize in the autoclave at 15 pounds' pressure for twenty minutes.

There is sufficient complement-fixing substance in this 50 cc. of medium to fix the complement in 10 to 12 cc. of blood.

Bile Medium
(For typhoid group)

1. Ox bile	900 cc.
2. Glycerol	100 cc.
3. Peptone	20 gm.

Combine 1, 2 and 3 and dissolve by heating over a water bath. Place in small flasks or bottles and autoclave.

Brilliant Green Lactose Bile

1. Peptone	10 gm.
2. Lactose	10 gm.
3. Distilled water	not more than 500 cc.

Dissolve 1 and 2 in 3. Add 200 cc. of fresh ox bile or 20 gm. of dehydrated ox bile dissolved in 200 cc. of distilled water. The solution of dehydrated ox bile shall have a pH between 7.0 and 7.5. Make up with distilled water to approximately 975 cc. Adjust the reaction to a pH reading of 7.4. Add 13.3 cc. of a 0.1 per cent solution of brilliant green in distilled water. Add sufficient distilled water to make the volume 1000 cc. Filter through cotton. Distribute in fermentation tubes and sterilize. The reaction after sterilization (determined by potentiometric and not by colorimetric methods) should be not less than pH 7.1 and not more than pH 7.4.

Levine's Eosin—Methylene Blue Agar
(Standard for water analysis)

1. Distilled water	1000 cc.
2. Peptone	10 gm.
3. K_2HPO_4	2 gm.
4. Agar	15 gm.
5. Lactose, 10 gm. or 20 per cent, sterile solution	50 cc.
6. Eosin, yellowish, 2 per cent aqueous solution	20 cc.
7. Methylene blue, 0.5 per cent aqueous solution	20 cc.

Dissolve 2, 3 and 4 in 1 by boiling. Make up loss due to evaporation. Place measured quantities in flasks. Just prior to use, add to each 100 cc. of the melted agar, 5, 6 and 7 in the following amounts:

Lactose, 20 per cent, sterile	5 cc.
Eosin, 2 per cent aqueous	2 cc.
Methylene blue, 0.5 per cent aqueous	2 cc.

Mix thoroughly and pour into Petri plates. Allow to harden and incubate for sterility. All ingredients may be added, sterilized in bulk or in tubes, and then stored. Remelt and pour plates.

Use.—For the routine determination of organisms of the *coli-aerogenes* groups in water.

Note.—For the isolation of pathogenic organisms from feces it is essential to reduce the dye content to one-half of the above amounts, and to test each lot of medium for bacteriostatic action before use with known cultures of the *Eberthella* and *Shigella* organisms.

Endo's Medium
(Standard for water analysis)

(a) *Basic Stock Agar.*—

1. Beef extract	5 gm.
2. Peptone	10 gm.
3. Agar	30 gm.
4. Distilled water	1000 cc.

Place 1, 2, 3 and 4 in a container and weigh. Boil the ingredients until the agar is dissolved and make up the lost weight with distilled water. Place in a straight-walled vessel and autoclave at 15 pounds' pressure for fifteen minutes. Allow the agar to solidify; dump it, in mass, on a clean paper, cut the detritus from the bottom and discard. Melt the clean clear agar and adjust the reaction to between pH 7.8 and 8.2. Distribute in 100 cc or larger measured portions. Autoclave at 15 pounds' pressure for fifteen minutes.

(b) *Preparation of Plates.*—It is necessary to add to the basic agar definite quantities of lactose, certified basic fuchsin and C. P. sodium sulfite. Prepare a 10 per cent solution of basic fuchsin in 95 per cent alcohol, allow to stand twenty-four hours; decant and filter the supernatant fluid. This is the stock solution. Prepare a 20 per cent C. P. lactose solution and sterilize by the fractional method. When ready to make

plates, melt a known portion of the stock agar and to each 100 cc. add the following ingredients in the order given:

"	"	5 cc.
"	"	0 5 cc.
"	quantity of hot distilled water	0 125 gm.

After the addition of the sodium sulfite mix all ingredients thoroughly and pour into sterile Petri plates. Allow the agar to set at room temperature and place in the incubator overnight to harden. Remove and check to determine sterility.

Agar, Sodium Desoxycholate

1. Water	1 kg.
2. Peptone	10 gm.
3. Agar	12 to 17 gm.
4. Sodium chloride	5 gm.
5. Lactose	10 gm.
6. Ferric ammonium citrate	2 gm.
7. Dipotassium phosphate	2 gm.
8. Sodium desoxycholate	1 gm.
9. Neutral red (1 per cent solution)	3 cc

Dissolve the peptone in the water. Add sufficient sodium hydroxide to bring the reaction to pH 7.3 to 7.5. Boil for a few minutes and filter through paper. Add the agar to the peptone water; allow to soak for at least fifteen minutes. Melt the agar by boiling or autoclaving. To each liter of melted agar add about 6 cc. of 1 N sodium hydroxide; then add all of the other ingredients (except the neutral red) in the order given and as rapidly as possible. Titrate the medium to pH 7.3 or 7.5 and add the neutral red. (Use phenol red indicator for the titration.) Sterilize the medium in flowing steam. *Heat only long enough to kill the vegetative cells.* (For tubes with 10 to 15 cc. of medium, heating for fifteen minutes is sufficient.)

Selenite-F Enrichment Medium*

1. Sodium hydrogen selenite (anhydrous)	4 gm
2. Peptone	5 gm.
3. Lactose	4 gm.
4. Sodium phosphates (anhydrous)	10 gm
5. Water to make	1 kg.

* Tetrathionate Broth Base (Bacto), dehydrated, is an excellent enrichment medium for isolation of *Eberthella*, *Salmonella* and *Shigella*.

Mix and warm to dissolve the ingredients. Bring to boil; further sterilization is not necessary. Determine experimentally the exact proportions of monosodium phosphate and disodium phosphate which, together with the particular kind of peptone used and a particular lot or make of sodium selenite, will give a reaction of pH 7.0 to 7.1.

Russell's Double Sugar Agar

1.	1000 cc.
2.	40 cc.
3.	4 cc.

Mix the melted agar and the two sugars in the above proportions and adjust the pH to 7.2. Add 50 cc. of 0.02 per cent aqueous phenol red. Filter if necessary, tube and sterilize in the autoclave at 8 pounds for twenty-five minutes. Slant the tubes with a deep butt. Check the reaction with known strains of *E. coli* and *E. typhosa*.

Simmons' Citrate Agar

(For citrate utilization)

1. NaCl	5 00 gm.
2. MgSO ₄	0.20 gm.
3. (NH ₄) ₂ HPO ₄	1 00 gm.
4. Sodium citrate (2 H ₂ O)	2.28 gm.
5. Distilled water	1000.00 cc.
6. Agar	20 00 gm.
7. Bromthymol blue, 1.5 per cent alcoholic solution	10 00 cc.

Dissolve 1, 2, 3 and 4 in 5 and add 6. Heat to dissolve the agar, adjust the pH to 7.2 and then add 10 cc. of 1.5 per cent alcoholic solution of bromthymol blue. Filter through cotton, tube, and autoclave at 15 pounds for fifteen minutes. Slant with a deep butt. Check the reaction of the medium with known cultures of *E. coli*, *A. aerogenes*, *S. schottmuelleri*, and *E. typhosa* before using routinely.

Jordan's Tartrate Agar

1. Agar	20 gm.
2. Peptone	10 gm.
3. Sodium potassium tartrate	10 gm.
4. NaCl	5 gm.
5. Distilled water	1000 cc.
6. Phenol red, 0.2 per cent alcoholic solution	12 cc.

Add 1, 2, 3 and 4 to 5 and dissolve by heating. Adjust the pH to 7.4 and then add 12 cc. of 0.2 per cent alcoholic solution of phenol red. Tube in approximately 10-cc. quantities and autoclave at 15 pounds for fifteen minutes. Check the reaction of the medium with known cultures of *S. aertrycke*, *S. enteritidis*, *S. paratyphi* and *S. schottmuelleri* before using routinely.

Lead Acetate Agar

(For H₂S test)

1. "	100 cc.
2. "	4 cc.
3. "	4 cc.
4. Lead acetate (sterile, 0.5 per cent solution)	1 cc.

To 100 cc. of the sterile melted agar, add 2, 3 and 4 of the sterile Seitz-filtered solutions in the quantities given. Tube aseptically. Incubate to determine sterility. Check the reaction of the medium with known strains of *S. paratyphi* and *S. schottmuelleri*.

Dieudonne's Alkaline Blood Agar

(For *Vibrio comma*)

1. Nutrient agar	700 cc.
2. Blood, beef, defibrinated	150 cc.
3. KOH, 1 N solution.	150 cc.

Prepare nutrient agar and neutralize to litmus (about pH 6.8). Mix equal parts of 2 and 3 and steam in the Arnold sterilizer for thirty minutes. To 7 parts of the agar add 3 parts of the alkali-blood mixture. Pour Petri plates and allow to harden uncovered but protected by paper. Place strips of sterile filter paper between the dish and the cover to absorb the moisture and ammonia. Incubate for fifteen hours at 37° C. before using.

Carbohydrate Broth (For fermentation tests)

Before the addition of fermentable substances to broth it is necessary to render the basic medium sugar-free. Take 1000 cc. of infusion broth and inoculate with an actively growing culture of *E. coli*; incubate for eighteen hours at 37.5° C. Kill the organisms by boiling a few minutes. Place 20 to 30 gm. of purified talc in a large mortar and gradually add the broth while grinding, until all is thoroughly mixed. Filter through wet

sterility.

Note.—Some bacteriologists use free broth as a vehicle for the substances mentioned above may be chosen.

Lactose Broth (Standard for water analysis)

Lactose broth is prepared in the same general manner as nutrient extract broth with the addition of 0.5 per cent of lactose. The removal of muscle sugar is unnecessary as the beef extract and peptone are free from any fermentable carbohydrates. The reaction should be between pH 6.4 and 7. Sterilize in the autoclave at 15 pounds for fifteen minutes, limiting the total time of exposure to heat to one-half hour.

Clark and Lubs' Medium (For Voges-Proskauer and Methyl-red tests)

1. Peptone	5 gm.
2. Dextrose	5 gm.
3. Dipotassium phosphate (K_2HPO_4)	5 gm.
4. Distilled water	1000 cc.

Add 1, 2 and 3 to 4 and dissolve by heating. Filter through paper and replace the lost water. Tube in 10-cc. quantities and sterilize in the Arnold for twenty minutes on three successive days.

Bendick's Saccharose Peptone-water (For *Vibrio corima*)

To 1000 cc. of peptone solution, neutralized to phenolphthalein, add 1 gm. of anhydrous sodium carbonate. Boil and filter. Now add 5 gm. of

saccharose and 5 cc. of a saturated solution of phenolphthalein in 50 per cent alcohol. Tube in 10-cc. portions and sterilize in the Arnold for fifteen minutes on three successive days.

Peptone Solution (Dunham's)
(For indol test)

1. Bacto-tryptone (Difco)	10 gm.
2. Sodium chloride	5 gm.
3. Distilled water	1000 cc.

Dissolve 1 and 2 in 3 by heating. Adjust the reaction to pH 7.6. Filter if necessary. Tube in 10-cc. amounts and autoclave at 15 pounds' pressure for fifteen minutes. Alkaline Bacto-tryptone solution, pH 8 to 8.4, is an excellent medium for nitro-indol reaction for *V. comma*. Other peptones may be worthless.

Nitrate Broth
(For nitrate reduction test)

1. Peptone	10 gm.
2. Potassium nitrate (KNO_3) nitrite-free	1 gm.
3. Water, distilled, ammonia-free	1000 cc.

Dissolve 1 and 2 in 3 by heating. Filter through paper. Tube in 10-cc. portions and sterilize in the Arnold for twenty minutes on three successive days.

Bromcresol Purple Milk

Remove the cream from sweet milk. Heat the comparatively fat-free portion in a cylinder in the Arnold sterilizer for twenty minutes. Re-skim the fat from the milk and to each liter add 40 cc. of a 0.04 per cent aqueous solution of bromcresol purple. Tube in 10-cc. quantities and sterilize for twenty minutes in the Arnold on three successive days. Incubate to determine sterility.

Loeffler's Medium
(For *C. diphtheriæ*)

Collect beef blood in sterile, large glass or porcelain-lined containers. Allow to clot. Break up the clot in a sterile glass container with a sterile glass rod. Mix the blood with 3 parts of distilled water, containing 1 per cent of sodium chloride. Gently raise the temperature to about 50° C. until the serum is firmly coagulated. Sterilize for twenty minutes in the Arnold on three successive days. After sterilization, paraffinize the cotton plugs and test for sterility.

Hiss' Serum-water Medium
(For fermentation tests)

To 1 part of clear serum add 3 parts of distilled water, mix and heat in the Arnold sterilizer for fifteen minutes to destroy ferments. Add 1 per cent of the desired carbohydrate dissolved in a small amount of hot water. To each 1000 cc. add 50 cc. of a 0.02 per cent aqueous solution of brom-

thymol blue. Tube and sterilize for twenty minutes in the Arnold on three successive days. Incubate to determine sterility.

Glycerol Agar

To 1 liter of melted meat infusion agar add 30 cc. of pure glycerol. Adjust the reaction to pH 7.2. Tube, autoclave at 15 pounds for fifteen minutes and slant.

Petroff's Medium

(For *M. tuberculosis**)

This medium is composed of meat juice, eggs and a minute amount of gentian violet.

1. *Meat Juice*.—In a cool place infuse 500 gm. of beef or veal in 500 cc. of a 15 per cent solution of glycerol in water; after twenty-four hours, place in a sterile press and collect the extract in a sterile container.

2. Immerse washed eggs in 70 per cent alcohol for ten minutes. Pick out with sterile tongs, flame, and break in a sterile container. Stir until well mixed and strain through sterile gauze into a sterile container. Add 1 part of meat juice to 2 parts of egg by volume.

3. Add 1 per cent alcoholic solution of gentian violet to make a final proportion of 1 to 10,000. Thoroughly mix the ingredients, tube, inspissate, and sterilize as in making Loeffler's medium. Ordinarily, 350 cc. of meat juice, 28 eggs and 10 cc. of 1 per cent alcoholic gentian violet will be needed for 1000 cc. of the medium.

Cystine Blood Agar

(For *P. tularensis*)

1. Beef or veal infusion broth	1000 cc.
2. Agar	15 gm.
3. Peptone	10 gm
4. NaCl	5 gm

Add 2, 3 and 4 to 1 and dissolve by heating. Adjust the reaction to pH 7.8. Autoclave at 15 pounds for thirty minutes and filter. Place in a flask and again autoclave at 15 pounds for fifteen minutes. Before use, add to the above:

5. Cystine or cystine hydrochloride	1 gm.
6 Glucose	10 gm.

To the basic medium add 5 and 6 and dissolve by heating in the Arnold, and then sterilize for thirty minutes. Cool to 50° C. and add 50 cc. of sterile horse blood. Tube aseptically in 10-cc. portions and slant. Incubate to determine sterility.

Noguchi's *Leptospira* Medium

1.	800 cc.
2.	100 cc.
3.	100 cc.
4. - distilled water)	10 to 20 cc.
} parts	

* For special selective media see Diagnostic Procedures and Reagents, American Public Health Association, 1941.

Combine the above sterile solutions and tube in 10-cc. amounts under aseptic conditions, or mix 3 and 1 and sterilize in the autoclave for twenty minutes. Cool to 55° C., add 2 and sufficient of 4 to color a faint pink. Tube in 10-cc. portions under aseptic conditions. Incubate at 37° C. to determine sterility.

Tryptone-Glucose-Extract-Milk Agar
(For milk analysis)

1. Agar	15 gm.
2. Beef extract	3 gm.
3. Tryptone	5 gm.
4. Glucose	1 gm.
5. Distilled water	1000 cc.

Dissolve by boiling over a free flame, make up volume lost, and adjust reaction to pH 7. Add 10 cc. of skim milk. Dispense measured amounts (100 or 200 cc.) in flasks, or place 10 to 12 cc. in test tubes. Autoclave fifteen minutes at 15 pounds.

Tellurite Medium
(For *C. diphtheriæ*)

Melt infusion agar or 0.2 per cent dextrose agar, 10 cc. in tube or larger measured quantity in flask, and cool to 50° C. For each 10 cc. of medium, add 1 cc. of citrated or defibrinated blood and 1 cc. of a sterile 2 per cent solution of potassium tellurite. Mix and pour into Petri dishes. An excellent tellurite medium may also be prepared by adding 5 cc. of Bacto-tellurite blood solution to 100 cc. of Bacto-dextrose proteose No. 3 agar, heating to 80° C., cooling to 50° C., and pouring plates.

Bismuth Sulfitc Agar (Wilson and Blair)
(For *E. typhosa*)

A. Agar Base.

1. Agar, granulated or powdered	20 gm.
2. Beef extract	5 gm.
3. Peptone	10 gm.
4. Water, hot, to make	1000 cc.

Dissolve the ingredients by autoclaving for fifteen minutes. Store in a refrigerator if not used at once.

B.

.	6 gm.
.	20 gm.
3. Dextrose	10 gm.
4. Disodium phosphate, anhydrous (Na_2HPO_4)	10 gm.
5. Water	200 cc.

Dissolve the bismuth ammonium citrate scales in 50 cc. of boiling water; the sodium sulfite in 100 cc. of boiling water; and the dextrose in 50 cc. of boiling water. Mix the solution of bismuth ammonium citrate and sodium sulfite; boil; then add the sodium phosphate while boiling. Allow the mixture to cool; then add the dextrose solution. Add water to make

up lost weight and store in a well stoppered pyrex vessel in a dark cupboard at room temperature.

C. Iron Citrate Brilliant Green Solution.

- | | | |
|---|-----------|----------|
| 1. Iron citrate (ferric citrate) | | 1 gm. |
| 2. Water | | 100 cc. |
| 3. Brilliant green, 1 per cent solution | | 12 5 cc. |

Dissolve the iron citrate in the water with heat and add the brilliant green solution. Store in a well-stoppered pyrex vessel in a dark cupboard at room temperature. To 1000 cc. of hot melted agar base, add with thorough mixing, 200 cc. of bismuth sulfite mixture and 45 cc. of iron citrate brilliant green solution. Pour immediately into porous-top Petri dishes, 15 to 20 cc. to each. Keep the plates at room temperature for one to two hours and then store in a refrigerator until required. It is advisable to use these plates within four days after preparation.

Note.—Bismuth sulfite agar (Wilson and Blair) may be prepared by using Bacto or other dehydrated products. Directions for preparation are found on the bottle.

Chocolate Agar

(For *Neisseria**)

1. Strip lean meat from 5 to 6 fresh beef hearts and grind.
2. For each 500 gm. of meat, add 1 liter tap water
3. Infuse in refrigerator overnight
4. Strain and squeeze through coarse gauze
5. Add 10 gm. proteose peptone No. 3 (Difco) per liter.
6. Heat to 50° C for one hour
- 7.
- 8.
- 9.
10. Boil lightly for ten minutes.
11. Decant measured quantities in flasks and autoclave at 15 pounds for fifteen minutes
12. Cool to 60° C, add 5 per cent human or horse blood, place in water bath and heat slowly to 80° to 85° C, rotating flask to produce even mixture. Cool to 55° C. and pour plates.

BIOLOGICAL PRODUCTS

A variety of products are required, of which part are purchased from commercial manufacturers, while others are prepared in the diagnostic laboratory. The products prepared locally include: (a) autogenous vaccines, (b) antigens for diagnostic tests, and (c) antisera used for the identification of bacterial species.

I. Autogenous Vaccines.—If autogenous vaccines are to be made, it is important to exercise great care throughout their preparation in order to insure the selection of the organism responsible for the infection, to avoid contamination with spore-forming bacilli, especially those which produce powerful soluble toxins, and to obtain a sterile bacterial suspension which can be safely administered to the patient. As many different methods have been advocated for the preparation of autogenous vaccines, this discussion will be limited to a few general principles.

* Preferred to proteose peptone No. 3 agar and hemoglobin.

1. **Selection of Bacteria.**—With the infected materials, such as pus or tissues, prepare Gram stained films and examine them microscopically to determine the types of organisms present. Inoculate plates of infusion and blood agar, and hormone broth or brain broth media. Incubate at 37° C. for twenty-four to forty-eight hours. Examine the plates for predominating types of colonies and make Gram stain preparations from each type. Also make Gram stain preparations from the broth cultures. Select isolated colonies of the type desired and transfer to plates to isolate pure cultures. Transfer the broth cultures to plates, if organisms are not similar to those already isolated on primary plates. After growing the isolated strains, make Gram stain to insure purity. Transplant the culture to desired media for the vaccine. Do not use spore-forming bacilli or any organisms which are obviously contaminants.

2. **Preparation of Suspensions.**—Bacteria, grown on solid media in tubes, may be suspended by adding to the cultures small amounts of sterile salt solution, in which they are emulsified by shaking or by agitation with a platinum loop. If broth cultures are used, the bacteria should be sedimented by centrifugation and resuspended in sterile salt solution. Rewash the organisms in salt solution to eliminate traces of toxic products. The suspensions should be quite heavy, and of a density comparable to tubes 5 to 8 of the nephelometer (1500 million to 2400 million). The bacterial suspension is placed in a sterile flask or bottle containing beads and thoroughly shaken to break up clumps of organisms. It is then filtered aseptically through several layers of gauze or through coarse filter paper held in a small funnel, to remove particles of culture media or other debris.

3. **Dilution and Dosage.**—The concentrated bacterial suspension is next diluted with 0.85 per cent saline to the desired strength, which for adults is 1000 million per cc. and for children 500 million per cc. With such concentrations, initial doses of 0.1 cc. will contain 100 millions and 50 millions, respectively. If desirable, still smaller amounts may be used, increasing the subsequent doses by 0.1 or 0.2 cc. If two or more organisms are present, separate suspensions may be prepared and diluted as above, and if desirable they may be combined.

4. Methods of Determining Bacterial Content.

A. Direct Count (Helber Chamber).

(a) Materials.—

- (1) Helber chamber with reinforced cover slip.
- (2) Sterile culture tubes.
- (3) Precision pipets 1 cc. and 10 cc.
- (4) Saturated ethyl alcohol solution of methyl violet 6 B.
- (5) Five per cent aqueous phenol solution.
- (6) Buffered salt solution containing 1 per cent formalin.
- (7) Sterile capillary pipets.
- (8) Microscope complete with lighting equipment.

(b) **Technic.**—For each day's work, prepare a staining solution consisting of saturated alcoholic methyl violet 6 B, 0.5 cc. and aqueous phenol solution 5 per cent 50 cc. Mix the reagents and filter through a close-grained filter paper until free from small particles. Set up 3 sterile test tubes, and add, in order, the salt solution; 9 cc., 2 cc., 3.5 cc. 1 cc. of the bacterial suspension.

pipet. Transfer 1 cc. to the second tube containing 2 cc. of diluent. Mix and transfer 1 cc. to the third tube containing 3.5 cc. diluent. Mix the third tube and add 0.5 cc. of the filtered phenolized methyl violet solution. This gives a dilution of 1 to 150 of the original vaccine. Heat the tube containing the mixture of vaccine and stain over the flame until it bumps slightly. Remove and allow to stand for three minutes.

with a well-mixed portion of the stained vaccine, and by capillary attraction exactly fill the chamber. Place the cell on the stage of the microscope and allow the preparation to stand for five minutes. Locate the center of the ruled field with the $1/6$ power objective. Place a drop of thin cedar oil on the cover slip, increase the light and with oil-immersion lens focus on the squares. Count the bacteria in 1 square, raise the lens slightly and continue the count until those near the under surface of the cover slip are included. Continue the process until 20 squares are counted. All bacteria in clumps must be counted or accurately estimated. Duplicate counts and accuracy are essential.

(c) *Results*.—Divide the total number counted by 20 to obtain the average for 1 square. The squares are $1/20$ mm. by $1/20$ mm. and the chamber is $1/50$ mm. deep. Multiply the average for 1 square by 20 by 20 by 50 by 150 by 1000 = number of bacteria per cc. in the original suspension. (Or multiply the average of 1 square by 3 billion.)

B. Nephelometer Method (McFarland).—This method can be used when the suspensions contain no coloring matter. For broth vaccines a nephelometer prepared with broth must be used. The bacterial suspensions are compared with various densities of barium sulfate in a series of test tubes.

(a) *Nephelometric Standards*.—Set up 10 uniform test tubes of hard glass, add 1 per cent chemically pure H_2SO_4 , and 1 per cent chemically pure BaCl in the following amounts:

TABLE 51

Tube	H_2SO_4 (cc)	BaCl (cc)	Density corresponds with bacterial suspensions of about (millions)
1	9 9	0 1	300
2	9 8	0 2	600
3	9 7	0 3	900
4	9 6	0 4	1200
5	9 5	0 5	1500
6	9 4	0 6	1800
7	9 3	0 7	2100
8	9 2	0 8	2400
9	9 1	0 9	2700
10	9 0	1 0	3000

The tubes are hermetically sealed and labeled serially from 1 to 10.

(b) *Technic*.—Place a measured quantity of the bacterial suspension in a test tube of the same size and color as those used for the nephelometer standards and, if necessary, dilute with a measured amount of saline to the density of the standard.

(c) *Results*.—Determine the approximate bacterial content and multiply this by the dilution factor. For example, if the vaccine was diluted 4 times and the density corresponded with Tube 3, the factor of that tube (900 million), should be multiplied by 4, giving an estimated bacterial content of 3600 million per cc.

5. **Sterilization.**—Vaccines may be sterilized by the addition of chemicals or preferably by heat.

(a) **Chemicals.**—Place a measured quantity of the desired dilution of the suspension in a sterile vaccine bottle and add tricresol to a final concentration of 0.5 per cent. One cc. of a 5 per cent solution is required for 10 cc. of vaccine. Close the bottle with a sterile rubber stopper, mix and incubate at 37° C. for forty-eight hours. Remove 1.5 cc. with a sterile syringe and test for sterility and toxicity as indicated below.

(b) **Heat.**—Place the bacterial suspension in a sterile vial and seal hermetically. Weight the vial with sheet lead and heat under water in a water bath at 60° C. for one hour. Remove from the bath, dilute to the desired strength and place in a sterile vaccine bottle. Add tricresol to a final concentration of 0.25 per cent. Cork, mix and remove 1.5 cc. with a sterile syringe for sterility and toxicity tests.

6. **Sterility and Toxicity Tests.**—Place 0.25 cc. of vaccine in a tube of infusion broth or into an agar pour plate. Place 0.25 cc. into a shake culture of dextrose infusion agar. Incubate for seven days at 37° C. and examine the cultures on the second, fourth and seventh days for growth. Inject a mouse with 1 cc. intraperitoneally and observe the animal.

7. **The Label.**—The label should show the name of the patient, type of vaccine, bacterial content per cc. and the date made.

II. **Antigens.**—A variety of antigenic substances are required either for the production of specific diagnostic antisera in animals or for use in testing the sera of patients for specific antibodies. The following outline is limited to certain antigens commonly prepared or used in diagnostic laboratories.

Bacterial cultures to be used for inoculation into animals or for stock agglutinating antigens should not only be carefully studied to insure their purity, but their normal or dissociative state should be determined, and they should be so treated that the desirable antigenic components will not be changed or lost. Since this requires considerable investigation of the cultures, it can best be carried out in the larger well-equipped laboratories. The need for greater attention to the dissociative state of cultures is well illustrated by the array of antigens afforded by old cultures of *E. typhosa* which may include the so-called smooth motile (O-H), smooth non-motile (O), rough motile (Ø-H) or rough non-motile (Ø) types. Each of these different dissociative types, which may appear spontaneously in any culture of typhoid, produces in animals antisera containing correspondingly different antibodies. Likewise it is known that variants with changed antigenic structure may be produced in pure cultures of many other species of bacteria. For a full discussion of antigenic variations see Arkwright,* Topley and Wilson,† and Topley.‡

1. **Selection of Cultures.**—The following methods may be used in selecting bacterial cultures of the desired types either in the so-called normal or variant states.

* ARKWRIGHT, J. A.: System of Bacteriology, London, Med. Res. Council, vol. 1, 1930.

† TOPLEY, W. W. C., and WILSON, G. S.: The Principles of Bacteriology and Immunity, Baltimore, William Wood & Co., 1937.

‡ TOPLEY, W. W. C.: Outline of Immunity, Baltimore, William Wood & Co., 1933.

(a) *Normal Smooth Type Cultures*.—The methods required for the
those of the colon-typhoid-dysentery groups.

Inoculate nutrient agar plates, pH 7.2 to 7.4, with the culture so that
hours the colonies will be well
a magnifying glass or, preferably,
aral typical smooth type colonies,
and from each colony make transplants to tubes of infusion broth, and to
plates of infusion agar, pH 7.2 to 7.4. After incubation examine the cul-
tures for homogeneous growth in broth and for typical smooth colonies
on agar. After selecting the normal smooth type growth, make daily
serial transfers in infusion broth and on infusion agar plates for six days,
followed by transfers twice daily for four days, keeping all the cultures for
observation during this ten-day period. If the cultures show normal char-
acteristics, prepare transfers on infusion agar and in broth, using sufficient
material to obtain the desired quantity of bacterial growth, and incubate
at 37° C. for eighteen hours. When using organisms of a motile species,
added precautions should be taken to select only motile organisms in each
of the serial transfers.

(b) *Non-motile Variant Cultures*.—Non-motile variants of normal smooth
motile species may be obtained as in the case of *Proteus* OX19, by the
selection of single colonies, or by the use of a medium which inhibits the
development of flagella.

Inoculate dry infusion agar plates, pH 7.2 to 7.4, with the culture to
insure well separated colonies and incubate at 37° C. for eighteen hours.
Examine the plates with a hand lens or dissecting microscope and select
several smooth colonies without a fringed edge. Transfer each colony to
infusion broth and to other dry agar plates. Incubate and examine the
broth for non-motile organisms, and the plates to determine the type of
colony formation. Select the culture showing a homogeneous growth in
broth, but containing non-motile organisms, and showing on plates the
desired smooth colony. Transfer material from the selected plate culture
to dry agar in Kolle flasks or bottles to secure the quantity of growth
desired. Incubate at 37° C. for eighteen hours. Old cultures are preferred
for the isolation of non-motile strains, for with recently isolated motile
species the procedure must be repeated several times. After obtaining a
rather stable non-motile strain it should be observed frequently to detect
any reversion to the normal motile type, and also should be agglutinated
with "H" antiserum to insure its freedom from flagellar antigen.

Non-motile strains may also be obtained from the normal motile ones
by cultivation on agar containing phenol in the proportion of 1 to 500.
Substitute the phenol agar for the infusion agar above and transplant
selected colonies from plate to plate, checking for loss of motility from
time to time with broth cultures. Suspensions of pure "O" antigens can
be prepared with motile species either by heating the organisms or by
treating them with alcohol. As a rule antigens prepared by these methods
are preferred.

(c) *Rough Variant Cultures*.—Rough strains either of motile or non-
motile species may be obtained by colony selection, either from old broth

cultures or from recently isolated strains that have been grown for about ten days on 0.5 per cent lithium chloride or 5 per cent sodium chloride broth. Transfer material from the stock culture to 0.5 per cent lithium chloride or to 5 per cent sodium chloride broth and incubate for ten days at 37° C. Inoculate several dry infusion agar plates pH 7.2 to 7.4, and select the roughest type colonies. Examine

colonies for transfer to infusion broth and to infusion agar plates. Incubate for eighteen hours and examine the broth for granular growth and the plates for rough type colonies. Select the broth cultures with the most granular sediment, and from the corresponding agar plate again transfer the roughest type colony to broth and to an agar plate. When a rough culture is secured, all of the colonies on agar should be rough, and the broth completely sedimented after twenty-four hours. It should also spontaneously agglutinate in 0.8 per cent saline. There should be no agglutination with smooth "O" antiserum. Transplant to infusion broth and infusion agar in Kolle flasks or bottles to secure the quantity of growth required and incubate for eighteen hours at 37° C. If a motile species is desired, select a motile rough strain in the primary broth culture and re-check it for motility on the final culture.

The antigens of smooth non-motile organisms of the enteric group have been referred to as "O" or "somatic" antigens, while the antigen associated with the flagella of the motile species is referred to as "H" or "flagellar" antigen. Thus, the normal smooth non-motile species contain only "O" or body antigen; while the normal smooth motile organisms contain both "H" and "O". The "O" antigen may consist of one component as in *E. typhosa* or it may consist of several antigenic components as in *S. paratyphosae*. The "H" antigen may be monophasic as in *E. typhosa* or diphasic as in *Salmonella schottmuelleri*.

2 Preparation of Suspensions.—After growing the culture on infusion agar or in infusion broth for eighteen hours at 37° C., standard suspensions are prepared by methods which will preserve the desired antigenic com-

posed of the suspension is stirred through a fine gauze, into a sterile flask, to remove gross particles of agar and large clumps of bacteria. It is then diluted with 0.85 per cent saline until the density is similar to that of tube No. 3 of the nephelometer (McFarland) standard. Measure and store in flint glass bottles at 3° to 5° C. Broth cultures may be diluted and stored in the same manner.

3. Preservation of Antigenic Components.—Flagellar or "H" antigens of motile species may be prepared by adding 0.2 per cent C.P. formalin or 5 per cent chloroform to the suspensions of cultures either grown on agar or in broth. Mix and place in the refrigerator at 5° C. for four days before using. Somatic or "O" antigens of non-motile species may be made by heating at 60° C. for one hour, either broth cultures or suspensions of agar cultures. In preparing "O" antigens from motile species, the standard suspensions are heated at 90° C. for one hour and then preserved with 0.5 per cent phenol; or the antigen may be made by "Bien's" method. For the latter use the dense agar culture suspensions, before diluting. Measure the suspension in a cylinder and add to it, while stirring, an equal

volume of absolute alcohol. Cover and allow to stand at 37° C. for twelve to twenty-four hours. Shake the flocculent precipitate and store at room temperature for two hours. Add saline until the alcoholic content is reduced to 33 per cent. Place in sterile flint glass bottles and store in the refrigerator at 5° C. The suspension must be dense enough to allow for a dilution of 1 to 16 or more, before final use, in order to reduce the concentration of alcohol. Immediately before use, dilute this stock suspension with saline to a density of tube No. 3 of the nephelometer standard (McFarland).

These stock antigen suspensions can be used either in agglutination tests or for injection into animals in the preparation of antisera.

III. **Antisera.**—In the production of diagnostic antisera, horses, goats and rabbits are commonly employed, depending upon the quantity desired. For ordinary laboratory use, healthy, adult rabbits are commonly preferred.

1. **Inoculation and Dosage.**—Two methods of inoculation—subcutaneous and intravenous—are usually employed, depending upon the toxicity of the organism used as an antigen. If a toxic organism is used, a small primary dose may be given subcutaneously, followed, at three-day intervals, by small gradually increasing intravenous doses. The following schemes have been found satisfactory for the production of agglutinating sera.

TABLE 55

<i>Shigella dysenteriae</i>				<i>E. typhosa</i>			
1st day	25	million	subcut.	1st day	500	million	intraven.
2d "	25	"	intraven.	7th "	1000	"	"
5th "	50	"	"	14th "	1500	"	"
8th "	100	"	"	21st "	2000	"	"
11th "	250	"	"				
14th "	500	"	"				
17th "	1000	"	"				

About five days after the last injection, bleed the animal from the marginal ear vein, and determine the agglutinin titre of the serum with a standardized stock antigen. If the titre of the antiserum is sufficiently high, bleed the animal to death; otherwise continue the injections of antigen.

2. **Bleeding.**—The blood should be withdrawn and handled under aseptic conditions. Strap the animal to a board, clip the hair from the thorax and paint with tincture of iodine. Etherize, and with a large needle attached to a sterile 30-cc. syringe, puncture the heart and withdraw all or part of the blood. Place the blood in sterile flasks, slant and allow to coagulate at room temperature. Place the flasks upright in the refrigerator overnight and remove the serum. Centrifugalize the serum to remove all red cells.

3. **Preservatives.**—Pool the sera from all animals of the same lot, measure, and slowly add sufficient phenol until 0.5 per cent concentration is reached. Mix and store in the refrigerator. After standing for one month in the refrigerator, the final titration for the agglutinin content should be made.

4. **Titration.**—Set up a macroscopic tube agglutination test, with sufficient dilutions to reach beyond the end titre of the serum, using the stock antigen, and antigens of allied species. Incubate at 15° to 55° C. for eighteen hours and record the results. The highest dilution that produces complete agglutination is recorded as the titre of the serum.

5. **Records.**—For each lot of antisera, prepare a detailed record of the characteristics of the organism used in the preparation of the antigen, and of all steps in the production of the serum.

METHODS OF EXAMINATION

Collection of Specimens

Specimens for bacteriological examination are collected from select locations and handled aseptically to avoid extraneous contamination. Avoid collecting surface specimens following the use of strong antiseptics. . . . but the type depends on . . . or must be shipped. Specimens to be shipped must be sealed and packed to meet postal requirements.

I. Materials from the Eye.—Collect secretions from the conjunctiva with a cold sterile platinum loop or with a cotton swab during the acute stage of the disease. Sufficient material to prepare both smears and cultures should be obtained. Special material from the cornea, anterior chamber, or iris should be obtained by the ophthalmologist. Pus from styne should be collected by the attending surgeon. Immerse enucleated eye in boiling water or an antiseptic solution, open aseptically, and obtain material for smears and culture.

II. Materials from the Ear and Mastoid.—In infections of the external auditory canal the exudate is collected. In otitis media or mastoiditis, material is obtained from the middle ear by the attending surgeon.

III. Materials from the Nose, Sinuses and Nasopharynx.—Specimens from the nasal passages or from the nasopharynx are secured on a tightly rolled cotton swab.

IV. Materials from the Throat and Tonsils.—Throat specimens should be collected on cotton swabs, by using gentle pressure over the infected areas in order to secure materials from the deeper tissues. Both smears and cultures are then prepared. In infected tonsils, materials must be pressed from the crypts or sucked out by the use of a pump. Materials from removed tonsils should be carefully secured from the deeper portions of the crypts, using asepsis to prevent contamination.

V. Materials from the Maxilla.—In maxillary infections, the position of the apex by the radiograph and sterilize the mucosa with the following solution: 1 part tincture iodine, U.S.P., 1.5 parts acetone, and 0.5 parts of glycerol. Isolate the area with sterile cotton rolls. Under anesthesia reflect the mucoperiosteum with a sterile trephine, burr or chisel, remove the outer bone covering the apex. With a sterile curet, collect the periapical tissue for examination. If the tooth is to be extracted, cleanse the mouth, and remove deposits from the tooth. Dry the crowns of involved tooth and those adjacent to it. Sterilize the crowns and gingival tissue with the iodine-acetone-glycerol solution. Isolate the tooth with sterile gauze and moisten with sterile normal saline. In periodontal

infections, prepare the site of infection as above, then collect material from the depth of the pocket with a sterile capillary pipet, or by expressing a bead of pus from the pocket by pressure over the pocket, and collect on swab. Place this specimen in a sterile test tube, seal and label.

VI. Sputum.—For routine examination, collect in a waxed paper box soon after the patient awakes in the morning. If required for special examination, cleanse the teeth and gums thoroughly, wash the mouth with an antiseptic and rinse with boiled water. The patient is then instructed to cough and to expectorate directly into an acid-cleansed sterile container. Paper boxes or like containers are objectionable but are preferred to improperly cleansed glass containers that are not acid-washed.

VII. Blood.—Blood for bacteriological examination must be collected under aseptic conditions, by an experienced technician or preferably by a physician. The specimens are usually collected from the median basilic or median cephalic veins. Paint the skin over the area with tincture of iodine and allow to dry. Place a tourniquet around the upper arm, compress it gently and then fasten it. Instruct the patient to open and close the hand. Select a prominent vein, or if the veins are invisible locate one by palpation. Wash the skin with alcohol. Insert a sharp needle, attached to a dry sterile syringe, into the vein, following the direction of the flow of the blood. Withdraw 8 to 10 cc. of blood into the syringe, loosen the tourniquet, and remove the needle. Place an alcohol sponge over the puncture wound and have the patient or assistant compress it. Place 2 to 3 cc. of the blood into each of two flasks containing 100 cc. of selective media, and place the remainder in a large sterile test tube containing 1 cc. of a 0.2 per cent sterile solution of sodium citrate in physiological salt solution. Measured amounts of the citrated blood can be placed in plates and mixed with melted agar or other media as desired. For shipment, blood is collected as above and placed in bottles of sterile glucose broth, or, if typhoid is suspected, into bottles of sterile bile. Such culture bottles are labeled, packed and shipped in double containers.

VIII. Blood Serum.—Blood to be used for the collection of serum for agglutination, complement-fixation or precipitation tests may be obtained with a large sterile needle and a sterile tube. Allow from 5 to 10 cc. of blood to flow directly from the needle into the tube. Stand the blood specimen in an upright position, protected from dust and sunlight, until coagulation is complete. Loosen the clot from the side of the tube with a sterile wire or glass rod, and centrifugalize until the serum is free from red blood cells. With a sterile pipet, transfer the clear serum to sterile vials. Cork securely, label, pack and ship at once. Contaminated specimens deteriorate rapidly in warm weather and become unsuitable for satisfactory examination. For the determination of a virus 10 to 15 cc. of sterile serum or whole blood are required. Vacuum bottles are used for blood.

IX. Cerebrospinal Fluid.—Immediately after collection, a white cell count should be made. Centrifugalize and prepare films from the sediment. Inoculate suitable quantities on selective media. For determination of viruses, fresh fluid should be used in the inoculation of selective animals.

after which cultures are prepared according to the findings in the smears.

XI. Bile.—Bile collected by the surgeon at operation is placed in sterile tubes, from which cultures may be prepared. Specimens obtained by non-surgical drainage by using the Lyon technic must be free from free HCl. Cultures are prepared on selective media.

XII. Feces.—Specimens should be passed by the patient directly into a sterile container, such as a bedpan, basin, fruit jar, or Petri plate. A portion is then transferred with a sterile spatula to a sterile, wide-mouthed bottle or vial. Vials and metal spoons (item No. 44710 and 44000) are furnished. Specimens for shipment are preserved by (glycerol 30 cc., NaCl 0.42 gm., sterile water). Specimens are collected directly from the individual: after cleansing the anal region with green soap and boiled water, introduce a sterile swab moistened with sterile water into the anal orifice. In lesions of ulcerative colitis, specimens must be obtained by the proctologist.

XIII. Urine.—Catheterized specimens, using asepsis, should be provided. Specimens from infants must be collected directly from the cleansed urinary meatus. For carrier examinations the urinary meatus is cleansed and the specimen is passed directly into a sterile container. Centrifugalized sediment is used for smears, cultures, and animal inoculations.

XIV. Materials from the Urethra and Prostate.—Urethral pus is collected on a sterile cotton swab or platinum loop and films prepared. Cultures are prepared on selective media without delay. Prostatic secretions should be collected by the genito-urinary surgeon. If cultures are desired, these should be obtained in sterile Petri plates.

XV. Materials from the Vagina.—Pus from the urethra, cervix, Bartholin glands, or from the vagina should be collected on a sterile cotton swab. Specimens from children may be collected either by means of a slender swab inserted into the vagina or by irrigation with normal saline solution. The washings are centrifugalized and the sediment is used for examination. Smears are made and cultures prepared on selective media.

XVI. Materials from Wounds.—Specimens should be collected by the attending surgeon. Pus or fluid is collected on sterile swabs or with a platinum loop. If cultures are desired for anaerobic organisms, the request should so specify. Smears are made and cultures prepared on selective media.

XVII. Autopsy Materials.—To be of diagnostic importance, cultures should be obtained within one or two hours after death. Aseptic precautions should be used in obtaining any materials for examination.

XVIII. Disinfection of Discarded Specimens.—All specimens should be kept in the laboratory until growth has been obtained on culture or examination has been completed. Specimens should be autoclaved or placed in 5 per cent solution of cresol before being discarded.

MICROSCOPIC DEMONSTRATION OF BACTERIA

The bacteria in pathological specimens or from cultures may be demonstrated microscopically either in the living state or after fixation and staining. For the former the procedures commonly used include: (a) the hanging-drop method, and (b) the cover slip method, using either ordinary lighting or darkfield illumination.

I. Hanging-drop Preparation.—With a small applicator, ring the concavity of a hollow ground slide with vaseline. Place a small loopful of the bacterial suspension on the center of a clean cover glass and spread the drop slightly. Invert the slide and place its concavity over the cover glass so that the drop of fluid lies in the center. Reverse the position of the slide with its attached cover slip. Press the cover slip firmly in place to prevent evaporation. Place the slide on the microscope stage and with the low-power lens focus on the edge of the drop. Without changing the focus switch to the high-dry objective, locate the film and focus on the bacteria. The oil-immersion objective is used for still higher magnification. The growth of bacteria may be studied microscopically in such preparations by placing sterile fluid or solid culture media in the cell beneath the cover slip, and inoculating the media with microorganisms.

II. Cover Slip Preparation.—Place a small loopful of the bacterial suspension to be examined on a clean slide, and over it place a cover slip. Gently press the latter until it no longer floats; after movement has stopped examine microscopically.

III. Darkfield Method.—By the use of a special darkfield condenser which gives oblique illumination, objects which otherwise are micro-

known as a "funnel stop." Daylight is not sufficient for the apparatus, and therefore a special source of artificial light must be used. Illumination may be provided by a 6 volt 15 C.P. electric lamp which can be attached beneath the condenser, or by various other types of illuminators. Direct sunlight affords a satisfactory source of illumination.

1. Adjustment of Apparatus.—Remove the ordinary condenser and insert the darkfield condenser with its two lateral adjustment screws forward. Adjust the source of light until a bright ring or spot appears on the upper surface of the condenser. With the low dry objective, locate the top of the condenser, and the concentric ring. Manipulate the lateral adjustment screws until the ring appears directly in the center of the field. Remove the oil-immersion objective, unscrew the lens and insert the funnel stop with its small end toward the lens. Replace the objective in its original position.

2. Examination.—Secure clean slides 1.45 to 1.55 mm. in thickness, and clean flexible cover slips. Place a small drop of the fluid to be examined on the center of the slide and over this gently press a cover slip, avoiding bubbles. Ring the cover slip with vaseline. Lower the substage and place a drop of thin cedar oil, free of bubbles, on the upper surface of the condenser. Put the slide preparation on the mechanical stage and center the d by contact with the
Place a drop of cedar

isms, which should ap

IV. Vital Staining.

be brought out more clearly for microscopie study by incorporating with the bacterial suspension, certain staining solutions. For example, aqueous methylene blue may be added to the suspension to bring out the granules

or capsules in hanging-drop or cover slip preparations. Other vital stains, including neutral red, may be used.

V. Fixed and Stained Films.—Bacteria are commonly fixed on glass slides and stained before microscopic examination. A small loopful of the fluid specimen or of a saline suspension of the bacterial growth is spread thinly on the surface of a clean glass slide or cover slip. In preparing films from cultures of certain waxy organisms such as *Mycobacterium tuberculosis*, it is necessary to first paint the slide with an albumin fixative, so that the organisms will adhere to the glass.

Allow the film to dry and then fix it on the glass slide by passing the latter rapidly through the flame of a Bunsen burner several times. The fixed film may then be stained by one of the simple or differential methods already described, and examined with the microscope.

CULTIVATION OF BACTERIA

I. Selection of Media.—Examples of the selection of media used for

- broths.
- Diplococcus pneumoniae*: Blood agar, enriched broths.
Neisseria intracellularis: Chocolate agar,* blood agar, serum agar, enriched broths.
Neisseria gonorrhoeae: Chocolate agar,* blood agar, serum agar.
Hemophilus influenzae: Chocolate agar.
Corynebacterium diphtheriae: Loeffler's medium, blood agar, tellurite medium.
Brucella melitensis: Chocolate blood agar, liver infusion agar.
Brucella abortus: Chocolate blood agar, liver infusion agar.
Pasteurella pestis: Serum agar, infusion agar.
Pasteurella tularensis: Cystine blood agar.
Mycobacterium tuberculosis: Petroff's medium.
Bacillus anthracis: Extract agar.
Clostridia: Robertson's medium, glucose agar, bromocresol purple milk.
Eberthella, *Salmonella*, *Shigella* and *Escherichia*: Bismuth sulfite, S.S. medium, desoxycholate, eosin-methylene blue agar or others.
Vibrio comma: Peptone solution.
Yeasts and fungi: Sabouraud's medium (see fungi).

II. Inoculation of Culture Media.—Pure cultures of organisms may be isolated by one of the following methods using solid media:

1. Petri Plate Method.—The plates should be marked plainly with a wax pencil, giving the date and the name or number of the specimen.

(a) *Surface Inoculation.*—The inoculum may be spread over the surface of the solidified medium in one of the following ways: Place one or more loopfuls of the specimen under examination, depending upon number of organisms present, on the surface of the medium near the edge of the plate. With a sterile glass spreading rod or a platinum loop, spread the material uniformly over the surface, being careful to avoid breaking the

* For *Neisseria*.

medium. When using a loop for spreading, it is best to streak a small quantity of the material in parallel lines across the surface. The loop is flamed and the surface is again streaked at a right angle to the original line. This is repeated until the material is evenly distributed. In using swabs, they are streaked across one side of the plate, after which the spreading is completed with a loop as described above.

(b) *Pour Plates*.—With a sterile platinum loop or pipet, inoculate a small portion of the specimen into a tube of melted medium which has been cooled sufficiently to avoid killing the bacteria. Gelatin may be at 35° to 40° C., and agar may be at 40° or 42° C. After inoculation,

be careful to avoid the formation of bubbles. Transfer a loopful of this material to a second tube of melted medium, and continue the serial dilutions through several tubes if necessary to obtain isolated colonies. Pour the contents of each tube into a sterile Petri plate using care to avoid contamination. Spread the medium uniformly over the bottom of the plate and place on a level surface until it becomes solid. Invert the plates and place in the incubator. If it is desirable to estimate the numbers of viable bacteria present, inoculate the melted medium with measured amounts of the specimen accurately diluted in sterile water, broth or salt solution, as outlined under the bacteriological examination of water.

After incubation remove the plates and examine the colonies with a hand lens or preferably with a dissecting microscope. Select well-isolated colonies and with a platinum wire transfer to other media.

2. *Tube Method*.—Melted agar, in deep tubes, may be inoculated as indicated under the pour-plate method, and allowed to remain in the tubes. As this provides conditions of partial anaerobiasis at the bottom of the tubes, the method is often useful for the isolation of facultative anaerobes. If the specimen contains bacteria in the spore stage, these may be isolated more readily by heating the material at 80° C. for thirty minutes to destroy the vegetative forms. After incubation of the inoculated tubes, flame the plug, heat the tube in a small flame enough to soften the surface of the agar and then heat the bottom of the tube until the cylinder of agar is expelled into a sterile Petri plate. With a sterile spatula cut the agar into sections in order to select isolated colonies for study and transfer. If desired the cylinder of agar may be removed without heat by washing the tube in alcohol and then filing and breaking it into a sterile Petri plate.

Various other methods of obtaining pure cultures of bacteria include the use of selective bactericidal substances, agglutinating serum, selective media, the inoculation of susceptible animals and the single-cell culture methods.

III. *Incubation*.—The cultures are grown at constant temperatures in incubators, which are usually double-walled, water-jacketed, supplied with automatic thermo-regulators and heated either with gas or electricity. A vessel containing water should be kept in the incubator to provide moisture and prevent drying of the cultures.

1. *Temperature*.—As most of the pathogenic bacteria grow well at a temperature of 37° C., the incubator is usually adjusted at this point. The length of incubation required for different species varies from a few hours to several weeks. Many saprophytic bacteria and fungi grow best

at low temperatures and may be incubated at from 20° to 30° C. Cultures in gelatin may be incubated at temperatures below the melting-point of the medium (about 20° C.), either in a "cold" incubator or at room temperature. Or, if the organisms are fastidious in their temperature requirements, gelatin cultures may be incubated at 37° C. and later chilled to determine whether the medium will again solidify.

IV. Atmospheric Conditions.—Most pathogenic organisms, being aerobes, will grow readily when incubated under ordinary atmospheric conditions. Others, including the microaerophiles and the anaerobes, require the use of special methods, some of which will be considered below. The anaerobic bacteria differ greatly in their atmospheric requirements. Some are able to develop under conditions of reduced atmospheric oxygen while others, the obligate or strict anaerobes, will not grow in the presence of even small amounts of free oxygen. Of the many methods available the following have been found to be useful.

1. **Oxygen Expulsion.**—Certain facultative anaerobes grow well in the lower levels of long tubes of solid or fluid media containing a carbohydrate or a small piece of fresh sterile animal tissue, provided the medium has been boiled previously to drive off the absorbed oxygen, and has been rapidly cooled and covered with a protecting layer of sterile petrolatum. Such media, which should be inoculated with a long platinum wire or capillary pipet are obviously more suitable for the maintenance of pure cultures than for the isolation of individual bacteria from contaminated mixtures. For the latter purpose, plates are preferred.

2. **Oxygen Exhaustion.**—After placing the inoculated culture media in an air-tight Novy jar or desiccator, the air in the container may be exhausted by means of a vacuum pump. This method is not recommended as it is almost impossible to maintain satisfactory anaerobic conditions with it. The changed surface tension may interfere with growth; also the medium dries very rapidly.

3. **Oxygen Displacement.**—Place the culture in a Novy jar and replace the atmospheric oxygen by hydrogen or some other non-injurious gas. Connect the hydrogen supply to the intake of the jar and allow the gas to flow for about ten minutes, after which the stopcocks are closed and the jar placed in the incubator. Precautions should be taken to avoid explosion while using this method.

4. **Oxygen Absorption.**—One of the simplest of these procedures is based on the fact that pyrogalllic acid, when placed in an alkaline solution, will absorb large amounts of oxygen. The culture tubes or plates may be placed inside a desiccator or jar in the bottom of which has been placed 1 gm. of pyrogalllic acid for each 100 cc. of air space. Rapidly add to the pyrogalllic acid a 10 per cent aqueous solution of NaOH or KOH (10 cc. for each gm. of pyrogalllic acid) and quickly seal the jar. Many methods have been devised for using this procedure, including the Büchner method in which a small culture tube is sealed inside a larger tube containing the reagents; and Wright's method in which the chemicals are placed in the same tube with the culture, from which they are separated by a tight cotton stopper.

5. **"Brown Jar" Method.***—A modified "Brown Jar" method is recommended as most satisfactory for work with anaerobic bacteria. Apparatus

* BROWN, J. H.: Jour. Exp. Med., 33, 677, 1921.

of this type may be obtained from laboratory supply houses. Similar apparatuses, such as the Fildes-McIntosh jar or Brewer's modification, may be also used.

(a) *Precautions.*—In using these methods, there are several precautions which should be emphasized with a view to preventing possible explosions. First of all, each time before using the jar, make sure that the wires coming away from the spool are intact. Next, always be sure to displace, mechanically, most of the air in the jar by allowing the hydrogen to flow slowly through it before the lid is clamped down. Do not allow the spool to reach a red heat, as may happen with a new spool when hydrogen is run in rapidly. Cut down the flow of hydrogen, if necessary, turning the stop-cock entirely off for a few minutes to allow the spool to cool. Make sure there are no loose electric connections, and use the current only when necessary. It is frequently necessary to use current only toward the end of the process. Do not clamp the lid on until the jar has been operating a while. If most of the atmosphere has been replaced mechanically, as described, and the lid is not clamped down immediately, any overlooked defect in the spool and its wires which might result in a spark before the last of the oxygen is exhausted, will cause only a very minor explosion. When handling the jar while it is in operation, take the precaution to cut off the current.

6. *Methods for Increasing CO₂ Tension.*—Certain organisms, including *Brucella abortus* and *Neisseria intracellularis*, grow best, especially on primary isolation, in an atmosphere containing about 10 per cent carbon dioxide. Such conditions may be obtained by one of the following methods:

Wadsworth's* method is described as follows: To produce an atmosphere containing approximately 10 per cent of CO₂, use a museum jar approximately 5 inches in diameter and 8 inches in height (inside measurements), which can be tightly sealed. Put the plates or tubes in the jar, then place 0.6 gm. of Na₂CO₃ in the bottom of the jar. Pour over it a mixture of 1 cc. of H₂SO₄ and 10 cc. of water. When the reaction begins to subside, place an air-tight cover on the jar. (These amounts are calculated for a jar of approximately 2½ liters.)

Candle Jar.—This method has been used for a number of years and has given very satisfactory results. It is recommended because of its simplicity and availability. Any jar with a tightly fitting cover that will permit entrance of Petri dishes may be used. Museum jars or similar ones can be sealed with plasticene or vaseline. Screw-capped gallon jars can be sealed with vaseline. The jars are prepared by placing a moistened piece of cotton or gauze over the bottom. The half of a Petri dish is inverted over the moistened cotton or gauze. Cultures on dishes are inverted and placed in jar. A candle, 2 inches in diameter, is placed in the jar and then allowed to extinguish. The jar is then placed in the incubator.

SEROLOGICAL TECHNIC

Serological reactions including agglutination, precipitation, complement-fixation, and other antigen-antibody tests may be used either for the

* WADSWORTH, A. B: Standard Methods, Div. Lab. New York State Dept. Health, Baltimore, Williams & Wilkins Company, 1927.

identification of bacterial species or for the detection of specific antibodies in sera from infected individuals.

I. Agglutination Tests.—For the agglutination reaction, which is one of the most useful of the routine diagnostic laboratory tests, three things are required: (a) Serum containing specific agglutinins, (b) antigen consisting of suspensions of either living or dead bacteria and (c) an electrolytic solution such as 0.85 per cent sodium chloride. When such materials are mixed in the proper proportions and incubated, the organisms in the bacterial suspension become clumped, a condition which may be recognized either by microscopic or macroscopic examinations. To be of diagnostic significance, the reactions of serum

A. G. may be performed by mixing small amounts of diluted serum with saline or broth suspensions of bacteria on glass slides or cover glasses, and observing the reactions either with the microscope or, if the suspensions are sufficiently opaque, with the unaided eye. Such examples are the Widal reactions and the Huddleson technic for *Brucella*.

1. The Detection of Agglutinins in Serum.—Macroscopic Slide Test (Huddleson).—An example of a macroscopic "slide test" is afforded by the Huddleson test which is used for the detection of *Brucella* agglutinins in sera of bovine or human origin. The materials required include: An illumination box on which can be fitted a piece of window-glass of double thickness, ruled in 5 lines of 1 inch squares; pipets of 0.2 cc. capacity graduated to 0.01 cc.; clean toothpicks; the bacterial antigen and the specimens of serum to be examined.

The bacterial antigen is prepared as follows: Grow a readily agglutinable strain of *Br. abortus* on liver infusion agar for seventy-two hours at 37° C and remove the growth in the smallest possible amount of 12 per cent NaCl solution containing 0.5 per cent phenol. After filtering through cotton add to each 100 cc. of the bacterial suspension 0.01 cc. of saturated aqueous gentian violet solution. Boil this slowly for ten minutes, refilter through cotton, cool and adjust the reaction to pH 6.8.

Standardize the antigen with known positive and negative sera as follows: Place 5 small numbered tubes in a rack, and add to each 0.5 cc. of antigen followed in series by 0.1, 0.2, 0.3, 0.4 and 0.5 cc. amounts respectively, of 12 per cent NaCl solution. Mix and test each of the 5 antigen dilutions with 3 sera, one having an agglutinin titre of 1 to 500 or 1 to 1000, another with a titre of 1 to 25 and the third a negative control. Use 1 drop of antigen to 0.08, 0.04, 0.02, 0.01 and 0.004 cc. amounts of each serum. The antigen dilution which fails to clump in the negative serum but is completely agglutinated by 0.08 cc. of the 1 to 25 serum and by 0.004 cc. of the 1 to 500 serum, is selected for use in the test.

The test is performed as follows: Prepare the specimens of serum by removing all cells. With a clean 0.2 cc. pipet place 0.08, 0.04, 0.02, 0.01 and 0.004 cc. amounts of each specimen respectively on 5 squares of the ruled glass plate. Add to each of these amounts of serum, 1 drop of the bacterial antigen, and mix with a clean, wooden toothpick. The dilutions are now 1 to 25, 1 to 50, 1 to 100, 1 to 200, and 1 to 500. Tilt the glass slowly back and forth for two minutes, adjust over the lighted reading-

box and examine with the unaided eye or with a hand lens for evidence of agglutination.

2. **The Identification of Specific Bacteria.**—Glass slide agglutination tests may also be used for the rapid presumptive identification of bacteria isolated from patients. They are commonly used to save time during the examination of large numbers of cultures as for example, in surveys for the detection of carriers of meningococci or cholera vibrios. However, such "spotting" tests are of presumptive value only and should always be followed by more accurate methods of identification.

B. Macroscopic Test Tube Methods.—The macroscopic tube methods are generally preferred for agglutination tests as they make possible the use of larger quantities of the reagents thus decreasing the chance of error. In some laboratories the reagents are measured by the drop method, but the use of accurately calibrated pipets is recommended.

1. **Reagents.**—As already indicated the necessary reagents include a uniform carefully standardized saline suspension of bacteria and measured amounts of the serum, so diluted as to cover the significant or diagnostic agglutination range. In examining a patient's serum for specific agglutinins the bacterial antigen must be prepared from stock cultures known to give satisfactory results with known positive serum. When the test is used for the identification of bacterial cultures, select specific agglutinating serum of which the agglutinin content and titre have been previously determined.

2. **Technic.**—Various methods may be used for performing such tests, but the following procedure is simple and has been found to be generally satisfactory. Place a series of 10 or more small test tubes in a metal rack. Place in the first tube 0.9 cc. of 0.85 per cent NaCl solution and in each of the remaining tubes 0.5 cc. To the first tube add 0.1 cc. of the serum, either undiluted or diluted as desired according to the titre. Mix the contents of tube No. 1 and from it transfer 0.5 cc. to tube No. 2. Mix the latter and similarly transfer 0.5 cc. to the next tube and so on throughout the series until the next to the last tube is reached. After mixing the contents of the latter tube discard 0.5 cc. Use the last tube, which contains only saline, as a control. The serum dilutions should extend beyond the titre of the serum. Add to each tube 0.5-cc. amounts of the bacterial suspensions which should be so opaque that the reactions can be easily seen. The final dilutions of serum are now 1 to 20, 1 to 40, 1 to 80, 1 to 160, 1 to 320, 1 to 640, 1 to 1280, 1 to 2560, 1 to 5120, and 1 to 10,240 respectively. Shake the tubes and incubate in a water bath at 37° C. or at 55° C., depending on the bacterial species used. The water level should be slightly lower than the surface of the fluid in the tubes. The results may be recorded after two hours, but the final readings should be made after standing eighteen to twenty-four hours in the water bath or refrigerator. The saline control tube should show no clumping. When the organisms are completely clumped and sedimented the reaction is considered as "double plus" (++) , when only half the organisms are agglutinated the reaction is recorded as "single plus" (+), and when fewer organisms are clumped the result is reported as "plus-minus" (=). Report the result obtained with

to whether the agglutination is of the floccular or granular type.

II. Agglutinin Absorption Tests.—At times when the results obtained by direct agglutination tests are indefinite, due to cross-agglutination, it may be possible to identify either the unknown organism or the specific antibodies in the patient's serum by agglutinin absorption tests.

1. Identification of Bacteria.—To identify an organism "C" which has been shown to be antigenically related to known species A and B, the following procedure may be used.

(a) Make cultures of A, B and C on suitable solid media in Kolle flasks and prepare dense saline suspensions of each.

(b) Prepare 1 to 10 dilutions of known antisera specific for the organisms A and B.

(c) Place 10-cc. amounts of each diluted serum in each of 3 tubes, and add to these the dense suspensions of organisms A, B and C respectively. Mix well and incubate in a water bath at 45° C. for four hours, shaking occasionally; then place in the refrigerator overnight. Centrifugalize at high speed until the supernatant fluid is clear.

(d) From each of the tubes remove the supernatant fluid, which is composed of the absorbed sera, in dilutions of 1 to 20. Prepare further dilutions as in the macroscopic tube agglutination test and use these for agglutination tests with suspensions of organisms A, B and C respectively.

If the unknown organism C completely absorbs all the agglutinins in serum A, it is considered to be similar to organism A; if on the other hand it absorbs all agglutinins from serum B, this shows it as being similar to organism B.

2. Identification of Antibodies in Patient's Serum.—To identify the specific antibodies in a patient's serum which agglutinates two or more species of bacteria, dilute the serum and carry out agglutinin absorption tests as indicated above using suspensions of the various species indicated

organism may contain substances which will precipitate with bacteria-free filtrates of cultures of the homologous organism. Precipitin tests may be used for the identification of a variety of bacterial species and of a great range of protein substances including the blood and tissues of various animals and plants. An example of its application to the former purpose is afforded by tests for determining the types of pneumococci. The soluble products of the organisms, either in the sputum or urine of pneumonia patients, in the peritoneal exudate of infected mice or in the filtrates of old cultures or their saline extracts, give specific precipitates with anti-pneumococcic serum of appropriate type. The clear serum and antigen are placed in a small crystal-clear tube, taking care to layer one fluid above the other. Incubate the tubes at 37° C. for two to four hours and examine. A positive reaction is indicated by a white precipitate at the zone of contact. If the fluids are mixed, the precipitate will be distributed throughout and will gradually settle to the bottom of the tube.

Precipitin tests are used less frequently than agglutination tests in routine bacteriological diagnostic work.

IV. Complement-fixation Tests.—The technic of complement-fixation tests is given in Part I, Chapter XII, and in Part X, Chapter XLIII.

PENICILLIN DETERMINATIONS

I. Detection of Antibacterial Activity in Body Fluids—Seeded Blood Plate Method.*—1. Materials.—Pour ordinary blood agar plates and inoculate them with a twenty-four-hour broth culture of beta hemolytic streptococci. After the medium has become firm, punch out circular areas 5 mm. in diameter with a sterile cork borer. Store the plates in the refrigerator until needed for testing.

2. Procedure.—Pipet 0.05 cc. of the blood or other body fluid to be tested directly into one of the punched out areas, then incubate the plate at 37.5° C. for twenty-four hours.

A simple method of collecting blood for this test is to place a small drop of oxalate solution on the clean nail bed and then prick the skin through the drop of oxalate.

3. Result.—If penicillin is present there will be a zone of inhibition of growth surrounding the area. The extent of this zone is a rough measure of concentration of penicillin in the sample tested.

II. Determination of Penicillin in Body Fluids and Exudates.†—1. Materials and Reagents.—(a) *Standard Penicillin Solution.*—Dilute a solution of penicillin with 0.85 per cent sodium chloride to a final concentration of 20 units per cc. Keep in the refrigerator.

(b) *Plain Broth.*

(c) *Specimens.*—Withdraw samples of venous blood and allow it to clot in a sterile tube. Separate the serum and store it at 5° C. until the time of testing. Keep other specimens at the same temperature. If specimens are known to be contaminated sterilize by filtration through a Seitz filter.

2. Procedure.—Make serial dilutions of 0.2 cc. of serum or other sample with 0.2 cc. of plain broth. Set up a series of 3 to 14 dilutions in sterile serological tubes.

Set up a similar series of dilutions of the standard penicillin solution.

Add to each tube in both series 0.5 cc. of plain broth containing 1 per cent of red blood cells and from 1000 to 10,000 Group A hemolytic streptococci. Incubate for eighteen to twenty-four hours and then examine for growth.

3. Results.—In general, those cultures showing no hemolysis are sterile but this should be checked by streaking several dilutions on either side of the apparent endpoint on blood agar plates.

The concentration of penicillin in 0.2 cc. of the unknown is determined by comparison with the standard control.

* TAYLOR, HARVEY G., Lawson General Hospital, unpublished manuscript.

† HAMMELKAMP, CHARLES H., Commission on Acute Respiratory Diseases of the Board for the Investigation of Epidemic Diseases, United States Army, unpublished manuscript.

Our thanks are due Dr. Hammelkamp and Major Taylor for making available these methods.

MEDICAL BACTERIOLOGY

By LELAND W. PARR

THE material presented in this section is intended as a guide to the classification, isolation and identification of bacterial species of medical importance. These include pathogenic organisms known to be responsible for human infections, organisms of doubtful pathogenicity, non-pathogenic organisms commonly encountered as contaminants of pathologic specimens and certain organisms of significance in routine laboratory examinations of eating utensils, disinfectants, food, milk and other dairy products, shell fish, water and sewage.

CHAPTER XXV

CLASSIFICATION OF BACTERIA

THE classification of bacteria followed in this book is that given in Bergey's Manual of Determinative Bacteriology, 5th edition, Williams and Wilkins Company, Baltimore, 1939.

I. Nomenclature.—When bacteria are assigned a scientific name consisting of genus and species the nomenclature used in Bergey's Manual should be given preference and printed in italics, the name of the genus beginning with a capital letter and that of the species with a lower case character; *e. g.*, *Mycobacterium tuberculosis*. Synonyms are permissible and

in italics; *e. g.*, the tubercle bacillus. The names of bacteria should be binary combinations consisting of the name of the genus followed by a single specific epithet; *e. g.*, *Escherichia coli*, not *Escherichia coli communis*. Many trinomials are present in the literature and to be rendered usable the epithets are hyphenated; *e. g.*, *Escherichia coli-communis*. A proposed new name should always be a binomial. No accepted list of abbreviations for genera exists. The initial alone may be used in papers or sections thereof in which the genus has been fully spelled out and where there is no possibility of confusing two genera beginning with the same letter. Thus out of context *E. coli* might be read as *Endameba coli*, as has actually happened, when it was supposed to mean *Escherichia coli*. In such a case confusion is avoided by writing as much of the genus name as is necessary to insure clarity of understanding; *e. g.*, *Esch. coli*, *Myco. tuberculosis*, *Br. abortus*.

II. Classification.—The Bergey Manual accepts as valid the descriptions of 1577 species of bacteria. These bacteria are distributed in 7 orders, 27 families, and 122 genera. Three only of the 7 orders contain bacteria of medical importance and among their 15 families 3, *viz.*, NITROBACTERIACEÆ, ACETOBACTERIACEÆ, and AZOTOBACTERIACEÆ are omitted because of lack of medical significance. Again not every genus among the 49 in the 12 remaining families is of medical significance. Helpful to a proper under-

standing of the relationship of bacteria to medicine is the further fact that contains species is the necessity thogenic micro- Representative species of the genera of importance in medical and sanitary bacteriology are listed in the following table which indicates the tribe, family and order of each.

MICROORGANISMS OF SPECIAL IMPORTANCE FOR MEDICAL AND SANITARY BACTERIOLOGY,
CLASSIFICATION OF BERGEY'S MANUAL, 1939

CLASS: SCHIZOMYCETES			
Family	Tribe	Genus	Representative species
<i>Order: Eubacteriales</i>			
II Rhizobiaceae*	...	Chromobacterium	C violaceum (B. violaceus)
		Alcaligenes	A. faecalis (B. alcaligenes)
III Pseudomonadaceae	Spirillum	Vibrio	V. comma (Cholera vibrio)
	Pseudomonadeae	Pseudomonas	P. aeruginosa (B. pyocyaneus)
VI Micrococcaceae	...	Micrococcus	M. luteus
		Staphylococcus	S. aureus
		Gaikya	G. tetragenus (M. tetragenus)
		Bacina	S. luteus
VII. Neisseriaceae	...	Neisseria	N. gonorrhoeae (Gonococcus)
			N. intracellularis (Meningococcus)
VIII. Parvobacteriaceae	Pasteurellae	Pasteurella	P. pestis (B. pestis)
			P. tularensis (B. tularensis)
		Malleomyces	M. mallei (Glanders bacillus)
	Bruceellae	Bruceella	B. melitensis (M. melitensis)
	Hemophilae	Hemophilus	H. influenzae (B. influenzae)
			H. pertussis
		Noguchia	N. granulosa (B. granulosa)
		Dialister	D. pneumonitis (B. pneumonitis)
IX. Lactobacteriaceae	Streptococcae	Diplococcus	D. pneumoniae (Pneumococcus)
		Streptococcus	St. pyogenes (Streptococcus)
	Lactobacillae	Lactobacillus	L. acidophilus (B. acidophilus)
X Enterobacteriaceae	Escherichiae	Escherichia	E. coli (B. coli)
		Aerobacter	A. aerogenes (B. aerogenes)
		Klebsiella	K. pneumoniae (K. pneumoniae)
	Serratiae	Serratia	S. marcescens (B. prodigiosa)
	Proteae	Proteus	P. vulgaris (B. proteus)
	Salmonellae	Salmonella	S. schottmulleri (B. paratyphus B)
		Eberthella	E. typhosa (B. typhosa)
		Shigella	S. dysenteriae (B. dysenteriae, Shiga)
			S. paratyphenteriae (B. dysenteriae Flexner)
XI. Bacteriaceae	...	Listeria	L. monocytogenes (B. monocytogenes)
		Actinobacillus	A. lignieresii (B. lignieresii; B. ligneri)
		Bacteroides	B. fragilis (B. fragilis)
		Eubacterium	E. plasma-vincentii (B. fusiformis)
XII. Bacillaceae	...	Bacillus	B. anthracis
		Clostridium	C. tetani (B. tetani)
<i>Order: Actinomycetales</i>			
I Mycobacteriaceae	...	Corynebacterium	C. diphtheriae (B. diphtheriae)
		Mycobacterium	M. tuberculosis (B. tuberculosis)
II. Actinomycetaceae	...	Leptotrichia	L. buccalis
		Erysipelothrix	E. rhusiopathiae (Bact. erysipelas suum)
		Actinomyces	A. hominis
			A. naadarii
<i>Order: Sparocetales</i>			
I Sparocetaceae	...	Bacterium	B. recurrentis (Sp. chlamydiae)
			B. vincentii (Sp. vincentii)
		Treponema	T. pallidum (Sp. pallidum)
		Leptospira	L. interrogans (Sp. peritum)
			L. interrogans (Sp. peritum)
			L. interrogans (Sp. peritum)
			L. interrogans (Sp. peritum)
			L. interrogans (Sp. peritum)

* The numbers prefixed to names of bacteria are those of the complete Bergey classification.

CHAPTER XXVI

BACTERIA OF MEDICAL IMPORTANCE

By LELAND W. PARR

THIS chapter is devoted to a brief presentation of the important generic and specific characteristics of pathogenic bacteria and of certain non-pathogenic species commonly encountered in the examination of pathological materials. It also includes practical methods for the bacteriological examination of clinical specimens. The order in which the various groups of organisms are considered conforms to the sequence in which the genera were presented in the previous edition of this book.

DIPLOCOCCUS

Organisms of this genus are defined as "Parasites, growing poorly on artificial media. Usually uniform turbidity in broth. Cells usually in pairs and lanceolate in shape. Capsules well developed. Bile soluble. Produce diseases of the respiratory tract." The *Diplococci* are Gram-positive organisms with high fermentative powers, most strains forming acid from dextrose, lactose, sucrose and inulin.

Five of the seven species of *Diplococcus* listed are obligate anaerobes found in septic wounds, the digestive tract, mucous secretions and the buccal-pharyngeal cavity. They are relatively unimportant forms and may not ferment inulin or dissolve in bile. Of the two aerobic forms *D. mucosus*, named before the types of the pneumococcus were recognized, is believed by many to be nothing more than a type III pneumococcus. The species of real medical importance is *D. pneumoniae* (pneumococcus). It is serologically very heterogeneous including as it does more than thirty distinct and stable immunological types.

Diplococcus Pneumoniae



colonies of green-producing streptococci from which they may be differentiated by bile solubility, inulin fermentation and high pathogenicity for white mice. On Loeffler's serum slants pneumococci produce moderate growth appearing on the

surface as a delicate layer or as small dewlike drops. Growth on potato and gelatin is very slight if at all.

Broth.—In serum broth after twenty-four hours at 37° C. there is a diffuse turbid growth with slight deposit, increasing on prolonged incubation. No pellicle is formed.

Resistance 7 5 4 3 2 1

temperature

Survive in a

or drying.

from a number of sugars including dextrose, lactose, sucrose and inulin. Milk is acidified and usually clotted. Nitrates are not reduced. Indol is not formed. Gelatin is not liquefied. No soluble hemolysin produced. Soluble in bile.

determined

so-called somatic antigen in the cell substance of the coccus common to all pneumococci. Since the somatic antigen common to all pneumococci resides in the cell body, naked pneumococci are all serologically alike but the usual pneumococcus from the body has abundant capsule and this determines the antigenic differentiation.

Pathogenicity.—No filterable toxin. Pathogenic for man, and many laboratory animals. The white mouse is highly susceptible, the rabbit less susceptible and the

tant changes found in variants are: Loss of virulence, loss of power to produce a capsule, and loss of type-specific polysaccharide, leaving the common somatic, nucleoprotein antigen, so that the organism no longer has the property of type-specificity.

Examination of Clinical Materials.—There are at least thirteen common sources for the isolation of pathogenic bacteria from the human body, viz., blood, the eye, ear and mastoid, nose and throat, sputum and lungs, mouth and teeth, urine or genital discharge, the feces, gastro-intestinal tract or gall bladder, the skin, pus from wounds or lesions, transudates or

exudates and cerebrospinal fluid. Pneumococci may be recovered from all of these sources except the skin, the gastro-intestinal tract or gall bladder, and feces. The specimens ordinarily employed, however, in diagnosing pneumococcic infections are sputum, throat swabs and pleural exudate in pneumonia, cerebrospinal fluid in pneumococcus meningitis, peritoneal exudate in pneumococcus peritonitis and pus in otitis media and mastoiditis. Blood cultures in all pneumonic infections serve to show the presence or absence of bacteremia which has prognostic value and such cultures, when positive for pneumococci, give irreproachable material for confirming the results of sputum or pus examinations. Methods of examination of clinical materials are microscopic, cultural, pathological (the use of animals) and serological. All four methods are made use of in the search for pneumococci.

1. *Microscopic*.—Direct smears should be made of all materials to be examined except blood. The smears should be stained by Gram's method which will serve to indicate not only the presence of typical Gram-positive, lancet-shaped diplococci but their number per field, a point of importance for proper application of Neufeld's Quellung Reaction. A capsule stain may also be made and should reveal the distinct capsule of the pneumococcus, especially well marked in Type III, and rarely absent in films prepared from body source material.

2. *Serological Determination of Pneumococci and of Types of Pneumococci*.—(a) *Neufeld's Quellung Phenomenon*.—At the present time the Neufeld reaction is the most important test applied to determine pneumococcus types and incidentally to identify an organism as a pneumococcus. It is an antigen-antibody reaction. When an encapsulated pneumococcus is brought into contact with its specific anti-serum (rabbit) the antigen-antibody reaction is manifested by a swelling of the capsule. To obtain proper results obviously potent serums must be used and some account taken of quantitative relations. The "volumination" of the capsule in a positive Neufeld reaction is visualized by the use of methylene blue.

Simplification of the Neufeld reaction has been effected by a two-stage set-up. Polyvalent mixtures of the known specific type antisera are available as follows:

A—Types 1, 2, 7

B—Types 3, 4, 5, 6, 8

C—Types 9, 12, 14, 15, 17, 33

D—Types 10, 11, 13, 20, 22, 24

E—Types 16, 18, 19, 21, 28

F—Types 23, 25, 27, 29, 31, 32

(Types 26 and 30 are omitted because 26 has been identified with 6 and 30 with 15. Other newly recognized types are not usually included.)

The first step is to identify the "pool" to which the suspected pneumococcus belongs. The second step is to ascertain with which component type of the pool the coccus identifies, by testing with each serum making up that pool. It is thus possible by this procedure with not more than twelve "quellungs" to establish the type of a pneumococcus. Should the organism fail to react with any one of the six "pools" it remains to prove by bile solubility that it is a pneumococcus. If it is, and such strains are not numerous, it is either an "untypeable" form of a recognized type or one of the "higher" types for which serums are not yet in general use. Such cultures should be kept for further manipulation which may render them amenable to typing or permit their study with respect to a fuller understanding of the serology of the pneumococcus.

The Neufeld reaction is carried out with fresh sputum as follows:

1. Divide three clean slides into halves by wax pencil and label halves "A, B, C, D, E, and F."

2.

3.

4.

5.

6.

by us

to time over a period of thirty minutes.

7. Positive mixtures having been determined, repeat the test procedure with each serum contained in that mixture, until the positive type or types have been determined.

Recent work indicates that the test may also be applied to sputum dried on a slide. The test lends itself well to the study of cultures, peritoneal exudates of mice, cerebrospinal fluid and other materials. Too many pneumococci in the preparation may require more antibody than is present. An old broth culture in addition to containing too many pneumococci may contain much antigen in solution thus interfering with the test. The test serums may be impotent. Thus simple though the test appears to be it should be performed only by experienced workers who examine many specimens per year if the best results are to be obtained.

(b) *Agglutination and Precipitation Tests*.—Agglutination tests may be carried out by a microscopic slide technic. The Sabin slide agglutination may be applied to pneumococci obtained by culture or from the peritoneal exudate of mice. The macroscopic tube agglutination may also be performed with cultures or with mouse exudate and requires less skill in interpretation than do the Sabin or Neufeld tests. The chief use at present for agglutination tests is for confirmation of results obtained by simpler, quicker methods. Macroscopic methods particularly require large amounts of material but they do offer advantage in that they are usually set up to include a test for bile solubility which serves to help establish the presence or absence of pneumococci.

The Sabin slide agglutination is carried out as follows:

1. Put a minute drop of the culture or mouse peritoneal exudate on a clean glass slide, using a fine capillary pipet or a loop.

2. Add a loopful of serum (diluted if the label so directs) to 1 drop of culture, smearing the mixture evenly and thinly.

3. Add a drop of saline to a second drop of culture, and smear this mixture evenly and thinly.

4. Let the preparation dry in the air.

5. Fix by gentle flaming.

6. Stain with dilute aqueous fuchsin (10 cc. saturated alcoholic solution of basic fuchsin plus 90 cc. of water) and examine with the oil-immersion lens.

Where true type-specific agglutination occurs, organisms are found in clumps, often surrounded by masses of stained amorphous precipitate. Clumping caused by spontaneous non-specific agglutination can usually

be recognized by its occurrence in the control smear made with saline as well as in the preparations containing serum.

Precipitation tests may be carried out upon the precipitable substance found in sputum by the method of Krumwiede and Valentine; upon the precipitable substance which appears in urine in more than half the cases of pneumococcus pneumonia late in the disease; and upon the precipitable substance remaining in the peritoneal exudate of white mice infected with pneumococci after the mouse peritoneal washings have been freed by centrifugation of most of the cellular components of the exudate. Inasmuch as the specific substance which reacts with the antiserum is soluble it may of course be found in cerebrospinal fluid, culture filtrates or other fluids which have contained an appreciable number of pneumococci for some time. A precipitation test may sometimes be possible when an agglutination test cannot be done as for instance when the cocci present autolyze or become heavily contaminated with other bacteria and of course in such a medium as urine in which pneumococci are scanty in number or entirely absent.

3. Cultural.—Culture of specimens suspected of containing pneumococci is usually done from nasal or throat swabs, sputum, pus, peritoneal washings or mouse's heart blood on blood plates which are incubated at 37° C. for twenty-four hours and are examined on a

Neufeld tests.

infusion broth at least ten times the volume of blood taken for culture.

g serum or
s. Cultures
intravenous
urides par-
ses contain

para-aminobenzoic acid to ensure the growth of living pneumococci which may be present and might otherwise be suppressed by the drug. Janeway suggests that 5 mg. of para-aminobenzoic acid per 100 cc. of medium be used for this purpose.

Organisms which morphologically resemble pneumococci but do not react serologically should be isolated in pure culture and typing again attempted. If the type is not then identified the culture should be tested for its bile solubility as follows: In a small tube place 0.1 cc. of 10 per cent sodium desoxycholate solution or sterile ox bile and add 0.4 cc. of the culture to be tested. Shake the tube thoroughly. If the culture is a pure strain and the organisms are pneumococci the faint turbidity in the tube due to the suspended bacteria will completely clear. An additional cultural test is the fermentation of inulin (inulin serum water medium) positive for most pneumococcus strains, negative for most streptococcus cultures. It is to be noted, however, that if Gram-positive diplococci giving characteristic serological reactions are found they are usually accepted as pneumococci without further cultural study.

(For a discussion of the cultural method of Avery's "artificial mouse" see next section.)

4. **Mouse Inoculation.**—Mouse inoculation is carried out on those specimens which by microscopic examination show relatively few pneumococci or many contaminating organisms. It may be required either to obtain organisms for an original typing or to confirm a typing already done. White mice weighing 16 to 22 gm. are ordinarily used. Pneumococci are frequently abundant enough for typing within three to five hours. The technic of mouse inoculation is as follows:

A small portion of sputum, about 1 cc., preferably washed three times through three changes of sterile saline, is emulsified in a mortar with sterile saline. (A watery sputum cannot be washed.) The clumps of and expelling them from

In either case injection of about 1 cc. of the washed, emulsified sputum. If one of the more virulent types is present the mouse will usually show evidence of illness after five to eight hours. With a pipet withdraw some of the mouse's peritoneal exudate and make stained smears. If Gram-positive diplococci are present in numbers sufficient for a Neufeld Reaction (2 to 18 per microscopic field) this test may be carried out and a diagnosis made. Sub-culture to a blood agar plate may also be made at this time if indicated. If too few pneumococci are present the mouse may be examined again a few hours later or until the exudate shows sufficient pneumococci. If it is desired to do agglutination or precipitation tests the inoculation is not terminated until the peritoneal exudate obtained shows on staining roughly as many pneumococci as an eighteen-hour broth culture would reveal or until the mouse dies

The mouse is then killed, if it has survived to this stage, and the peritoneal and thoracic cavities opened aseptically. The exudate present and heart-puncture material may be stained for the Gram reaction of the diplococci and to demonstrate capsules. Indicated sub-cultures may also be made from these two sources. Then, using a sterile glass pipet wash the peritoneal cavity thoroughly with 3 to 5 cc. of sterile salt solution and place the washings in a centrifuge tube. Centrifugalize at low speed for about five minutes to throw down the gross cellular débris, which may be discarded. Transfer the supernatant suspension to a second tube and centrifugalize at high speed for fifteen minutes or until the supernatant is clear. With a pipet remove the supernatant fluid which may be used for precipitation tests. Resuspend the sediment in sufficient saline to give the desired turbidity for an agglutination test of pneumococci and use the suspension for the macroscopic tube-agglutination test and for the bile solubility test.

Mice are sometimes unattainable. A classical substitute is the Avery "artificial mouse" method. The sputum, or other specimen, is prepared as if for mouse inoculation. Between 0.2 and 0.3 cc. of the preparation are then inoculated into a tube of Avery's medium, which is essentially dextrose-blood-meat infusion broth containing a higher concentration of dextrose and at a slightly lower pH than is the case for such a medium as routinely used. This medium is particularly useful also as an enrichment for nasal or throat swabs to obtain sufficient organisms for typing or as preliminary to mouse inoculation. The "artificial mouse," being a good culture medium, is definitely less selective than the white mouse method in which mouse virulent strains of pneumococci will be recovered

even if originally present in very small numbers. The Avery method applied to a specimen containing many bacteria other than pneumococci may fail to reveal the *D. pneumoniae*, but failure is not apt to occur if the Avery tube is incubated carefully and examined after three to six hours following incubation and, if negative, at intervals thereafter. It should be remembered that the specimen for examination may not contain pneumococci. To demonstrate the other bacteria it may contain such as staphylococci, streptococci or *Hemophilus influenzae* culture on blood-agar plate is better than inoculation into either "mouse" and the making of such plates from suspect material is strongly recommended.

STREPTOCOCCUS

Organisms of this genus are defined as follows: "Cells spherical or ovoid, rarely elongated into rods, occurring in short or long chains or in pairs. Never arranged in packets and do not form zoögleal masses. Gram-positive, some decolourizing readily. Capsules not marked as a rule but well developed at times. Growth tends to be slight on artificial media and some species are aided by the addition of native proteins; isolated colonies are small and translucent; they may be effuse, convex or mucoid. Cultures are found which produce a rusty red growth in deep agar stabs. Certain strains form a yellow to orange pigment in starch broth. Action on blood is variable but characteristic changes are produced in stab cultures. Various carbohydrates are fermented with dextrorotary lactic acid as the dominant product. Carbon dioxide, volatile acids and other volatile compounds are produced in small quantities, if at all, from carbohydrate fermentation. Nitrites are not produced from nitrates and inulin is rarely attacked. Most species are aerobic and facultative anaerobic; many species are normally parasitic and some are highly pathogenic. None are soluble in bile."

"The strictly anaerobic streptococci, some of which produce gas and foul odors, are not yet completely defined and they may merit being separated in a new genus."

For more than sixty years streptococci have been the object of careful study. The importance of this group of bacteria is well known to the physician and its extent and complexity have been the despair and challenge of the bacteriologist. Efforts directed toward a taxonomic understanding of the streptococci have proceeded along different avenues of approach at various times. Our present understanding of the group is expressed in terms of three of these different methodologies.

Hemolytic Action.—Schottmueller showed that the various species of streptococci differ in their effect on blood. This observation made forty years ago is still valid. Its application enables us to recognize three groups of streptococci:

(a) Beta hemolytic streptococci sometimes called *Streptococcus hemolyticus*. These are the streptococci with a zone of clear hemolysis around each colony on blood agar. No intact red blood cells will be seen adjacent to the colony when it is examined with the microscope. If grown in blood broth these streptococci hemolyze the red blood cells. Hemolysis is due to a soluble streptolysin of which there are two different forms, viz., streptolysin O and streptolysin S.

(b) Alpha "hemolytic" streptococci, sometimes called *Str. viridans*. These are the streptococci whose colonies on blood agar characteristically are surrounded by a green pigmented area. In some cases the area seems to be a clear zone of hemolysis but in either case microscopic examination of the colony will show unhemolyzed red blood cells in the medium touching the colony. This is the streptococcus colony apt to be confused with that of the pneumococcus.

(c) Gamma streptococci. These streptococci produce no effect in the blood agar medium on or in which they grow. They are sometimes said to be "indifferent."

Biochemical Reactions.—At the present time this heading will include a variety of fermentations, tolerance limits for pH, heat, salt, dyes and so on. The reactions utilized are not of equal importance for all streptococci. Thus among the beta hemolytic streptococci the fermentations of trehalose and sorbitol, the ability to hydrolyze sodium hippurate and the resistance to inhibition by methylene blue are the most important reactions. In another group, the viridans streptococci, the splitting of esculin, the hydrolysis of starch and the fermentation of lactose are important.

Serological Groups and Types.—Streptococci are now classified into "Groups" by means of the precipitin reaction using as antigens extractions from the streptococci containing serologically active and specific polysaccharide. This work was slow to develop because group differentiation did not appear by this technic in testing streptococci recovered from the more dramatic of the human streptococcus-incited diseases. This was because practically all such streptococci belong to one serological group (Lancefield A). The specific polysaccharide responsible for this grouping appears to be part of the streptococcus cell body whereas that involved in the pneumococcus is found in the capsule. Eleven groups of streptococci have been identified by Lancefield and other workers in this field (see Table 61). Most of these streptococci, but not all, are hemolytic. The specific carbohydrate in the viridans streptococci appears, in general, to be type specific, instead of group or species specific as in the hemolytic group. This results in a serologically heterogeneous group not readily amenable for purposes of taxonomy to the invaluable Lancefield methods so useful in dealing with most hemolytic strains.

Types, within the groups, particularly group A, have been demonstrated under the leadership of Griffith and procedures for typing streptococci by Griffith's slide agglutination test are available. The procedure is reminiscent of pneumococcus typing methods but has as yet very much less general use. It is invaluable in epidemiologic and chemotherapeutic studies but much less necessary for routine work. Two or even three different antigenic entities enter into typing. Of these one is the type-specific protein, M, an antigen primarily associated with virulence. Less important as a virulence factor but apparently more significant for the type reaction is the T substance. M and T are so definitely correlated that but little added complexity results from the fact there are two factors involved in type specificity. In addition a mixture of nucleoproteins are found in the streptococcal cells. These are called "P" and while "usually not type-specific, nor even species-specific in their serological reactions" must not be lost sight of in a study of specificity. Griffith has described

30 types of which four, Nos. 7, 16, 20 and 21, are now known not to be group A types. Others, however, have been added (see Table 62).

The streptococci may be divided into five divisions. A general picture of the four aerobic groups, called: the pyogenic group, the viridans group, the lactic group and the enterococcus group, is given in Table 56, taken from Sherman.

TABLE 56.—DIVISIONS OF THE STREPTOCOCCI (Sherman, Bact. Rev)

Division	Group or species	Lancefield group	Growth at		Growth in presence of		Strong reduction	Survival 60°C., 30 min. utes	Nil, from peptone
			Hemolysis	10° C.	45° C.	0.5 per cent NaCl	pH 9.0	0.1 per cent methylene blue	
Pyogenic	<i>S. pyogenes</i>	A	+	—	—	—	—	—	+
	<i>S. mastitidis</i>	B	+	—	—	—	—	—	+
	<i>S. equi</i>	C	+	—	—	—	—	—	+
	"Animal pyogenes"	C	+	—	—	—	—	—	+
	The "human C"	C	+	—	—	—	—	—	+
	"Minute hemolytic"	F	+	—	—*	—	—	—	+
	Group G streptococci	G	+	—	—*	—	—	—	+
	Group E streptococci	E	+	—	—	—	—	—	+
	Group H streptococci†	H	+	—	+	—	—	—	+
Viridans	<i>S. salivarius</i>		—	—	+	—	—	—	—
	<i>S. equinus</i>		—	—	+	—	—	—	—
	<i>S. bovis</i>		—	—	+	—	—	—	+
	Varieties of <i>S. bovis</i>		—	—	+	—	—	—	+
	<i>S. thermophilus</i>		—	—	+	—	—	—	—
Lactic	<i>S. lactis</i>		—	+	—	—	+	+	+
	<i>S. cremoris</i>		—	+	—	—	+	+	+
Enterococcus	<i>S. faecalis</i>		—	+	+	+	+	+	+
	<i>S. liquefaciens</i>		—	+	+	+	+	+	+
	<i>S. zymogenes</i>	D	+	+	+	+	+	+	+
	<i>S. durans</i>		+	+	+	+	+	+	+

* Indicates occasional variation from type reaction. Extremely rare exceptions not noted

† Group H streptococci, of doubtful status with respect to hemolysis, also fall between the "pyogenic" and "viridans" streptococci in physiological characteristics.

demic sore throat, erysipelas and other acute, if less specific, diseases of man. Members of this group may possess such agents of virulence as erythrogenic toxin, responsible for the rash and other symptoms in scarlet fever, fibrinolysin which dissolves the clot of normal human blood, leucocidin which is injurious to white blood cells, and the Duran-Reynolds "spreading factor" which alters the permeability of tissues.

The viridans group of streptococci contains numerous, important members characterized by the failure to produce complete hemolysis on blood agar, by their ability to grow at 45° C., and by the fact that they are not grouped by the serological methods of the Lancefield classification. That

these organisms are essentially mucous membrane organisms is attested to by their failure to grow at 10° C. Viridans streptococci produce disease in man, particularly subacute ulcerative endocarditis and to some extent arthritis, focal infections and abscesses. It is to be noted that streptococci in general frequently increase on mucous membranes or in lesions in the presence of other diseases which in the past gave rise to suspicion of etiological relationship often not confirmed. One such "species," *Str. morbilli*, is of the viridans group. In general green-producing streptococci are more frequently found on normal mucous membranes than are pyogenic forms although these last are by no means rare in the "normal throat." The characteristics of the viridans streptococci appear in Table 58.

TABLE 57.—ADDITIONAL CHARACTERISTICS PYOGENIC STREPTOCOCCI (SHERMAN, Bact. Rev.)

	Group of species														
	<i>S. pyogenes</i>			<i>S. equinus</i>			<i>S. agalactiae</i>			<i>S. pneumoniae</i>			<i>S. pyogenes</i>		
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Lancefield group . . .	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Active fibrinolysis . . .	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sodium hippurate hydrolyzed . . .	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Starch hydrolyzed . . .	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Esculin split . . .	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—
Growth on 40 per cent bile-blood agar . . .	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Gelatin liquefied . . .	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Milk curdled . . .	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Final pH in glucose broth . . .	6.0	4.8	4.5	4.2	5.4	4.8	5.0	4.6	5.3	4.4	6.0	4.6	4.8	4.2	5.0
Acid produced from:															
Arabinose . . .	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Maltose . . .	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose . . .	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose . . .	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose . . .	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Raffinose . . .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Inulin . . .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Glycerol . . .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Mannitol . . .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sorbitol . . .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Salicin . . .	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—

* For significance of asterisk see Table 56.

TABLE 58.—ADDITIONAL CHARACTERISTICS VIRIDANS STREPTOCOCCI (SHERMAN, Bact. Rev.)

	Species or variety				
	<i>S. salivarius</i>	<i>S. equinus</i>	<i>S. bovis</i>	Varities of <i>S. bovis</i>	<i>S. thermophilus</i>
Growth in 2 per cent NaCl . . .	+	+	+	+	—
Starch hydrolyzed . . .	—	—	+	—	—
Sodium hippurate hydrolyzed . . .	—	—	—	—*	—*
Esculin split . . .	—	+	+	+	—
Gelatin liquefied . . .	—	—	—	—	—
Milk curdled . . .	—	—	—	—	+
Final pH in glucose broth . . .	5.4-4.0	4.5-4.0	4.5-4.0	4.5-4.0	4.5-4.0
Acid produced from:					
Arabinose . . .	—	—	—	—*	—*
Maltose . . .	+	+	+	+	+
Sucrose . . .	+	+	+	+	+
Lactose . . .	+	—	+	+	+
Trehalose . . .	—	—	—	—	—
Raffinose . . .	—	—	+	+	—
Inulin . . .	—	—	—	—	—
Glycerol . . .	—	—	—	—	—
Mannitol . . .	—	—	—	—	—
Sorbitol . . .	—	—	—	—	—
Salicin . . .	—	—	+	+	—

* For significance of asterisk see Table 56.

Streptococci are characteristically organisms of the mucous membranes of man and animals. The lactic streptococci, however, occur in dairy products. They are characterized by having low minimum and maximum temperatures of growth, strong reducing action and a marked tolerance to methylene blue. These organisms are desirable bacteria functioning efficiently and well in the field of dairy bacteriology. Their characteristics found in Table 59 are chiefly helpful here in providing identification for a chance strain encountered in laboratory practice; they are not pathogenic.

TABLE 59.—ADDITIONAL CHARACTERISTICS: LACTIC STREPTOCOCCI (Sherman, Bact. Rev.)

	Species	
	<i>S. lactis</i>	<i>S. cremoris</i>
Ammonia produced from peptone	+	—
Growth at 40° C.	+	—
Growth in presence of:		
4 per cent NaCl	+	—
pH 9.2	+	—
0.3 per cent methylene blue in milk	+	±
Sodium hippurate hydrolyzed	±	—
Starch hydrolyzed	—	—
Esculin split	+	±
Gelatin liquefied	—	—
Milk curdled	+	+
Final pH in glucose broth	4.5–4.0	4.6–4.0
Acid produced from:		
Arabinose	±	—
Xylose	±	—
Maltose	+	±
Sucrose	±	±
Lactose	+	+
Raffinose	—*	—*
Inulin	—	—
Glycerol	—	—
Mannitol	±	—*
Sorbitol	—	—
Sabin	±	—

* For significance of asterisk see Table 56.

The fourth group of streptococci, the enterococci, are considered as having their origin in the animal intestine and it might be supposed that they would be a delicate group. From a study of Table 56 it will be evident that they have high resistance

low minimum and high maximum

fit them to survive but also to

The pyogenic and viridans streptococci do not develop colonies on extract agar but enterococci in fecal specimens grow out readily on Endo's agar. Gelatin liquefaction is noted for some of the enterococci and some of them are hemolytic and "groupable" as Lancefield Group D. They may enter occasionally into pathological conditions but without much significance. The characteristics of the enterococci are shown in Table 60.

The fifth group of streptococci is that containing the eight anaerobic species. These forms will not be considered here but the statement may be made that they have some pathological significance but in at least one instance (puerperal fever) this pathogenicity has been overemphasized while possibly in other conditions more warranted it has not been sufficiently recognized.

The pathogenicity of the serological groups of hemolytic streptococci is outlined, modified from Lancefield's Harvey Lecture, in Tables 61 and 62.

TABLE 60—ADDITIONAL CHARACTERISTICS, ENTEROCOCCI (Sherrman, Bact. Rev.)

	Species			
	<i>S. faecalis</i>	<i>S. liquefaciens</i>	<i>S. symyonicus</i>	<i>S. durus</i>
Lancefield group		D		
Hemolysis	—	—	+	+
Gelatin liquefaction	—	+	—	—
Strong reduction	+	+	+	—
Actively fibrinolytic	—	—	—	—
Sodium hip purate hydrolyzed	+	+	+	+
Starch hydrolyzed	—	—	—	—
Eucalin split	+	+	+	+
Milk curdled	+	+	+	+
Final pH in glucose broth	4.5-4.0	4.5-4.0	4.5-4.0	4.5-4.0
Acid produced from:				
Arabinose	—	—	—	—
Maltose	+	+	+	+
Sucrose	—	+	+	—
Lactose	—	+	+	+
Trehalose	+	+	+	—
Raffinose	—	—	—	—
Inulin	—	—	—	—
Glycerol	—	+	+	—
Mannitol	+	+	+	—
Sorbitol	+	+	+	—
Salicin	+	+	+	—

* For significance of asterisk see Table 56.

TABLE 61—PATHOGENICITY OF SEROLOGICAL GROUPS OF HEMOLYTIC STREPTOCOCCI (Lancefield, Harvey Lectures)

Animal source	Chief pathogenic groups	Occasionally pathogenic groups	Groups found but apparently non-pathogenic
Man	A	B, C, D, F, G, H	K, L
Cattle	B, C	A, G	D, E, H, L
Horse	C		
Monkey		A, G	C
Dog	G, L, M		C
Chicken	C	A ?	G
Swine		E, L	
Goat			
Sheep			
Fox		M	
Ferret			
Rabbit		A, B	
Guinea pig			
Mouse		A, B, C	

The groups and type specific antigens known at the present time are shown in Table 62.

TABLE 62—GROUP AND TYPE SPECIFIC ANTIGENS OF HEMOLYTIC STREPTOCOCCI (Lancefield, Harvey Lectures)

Group specificity		Type specificity			
serological group	Group specific "C" substances	Specific types recognised	Type specific substances		Substances chemically and immunologically distinct for each type
			Designated	Chemical nature	
A	.. .	At least 30	"M" "T"	Proteins Undetermined	
B	Polysaccharides immunologically distinct for each group	Several	"S"	Polysaccharides	
C		Several	No symbol	Proteins	
Remaining groups	Several	"S"	Polysaccharides	

Examination of Clinical Materials for Streptococci.—Streptococci may be encountered from every one of the sources of materials from the body

of man. They are only equalled in their widespread distribution in pathological material by the staphylococcus group. Most specimens, however, come from blood, sputum, throat, nose and vaginal secretions, pus, skin lesions and spinal fluid of infected individuals. In collecting specimens from the skin lesions of erysipelas, inject a small amount of sterile broth into the skin at the margin of the inflamed area and then withdraw the broth which usually contains the organisms.

1. *Microscopic*.—Smears on glass slides should be prepared from all material for examination (except blood) and stained by Gram's method. Look for Gram-positive cocci, arranged in long or short chains. Microscopic examination cannot differentiate the various species of streptococci nor is it always possible to exclude streptococci by such examination.

2. *Cultural*.—*The isolation of streptococci and the differentiation of these organisms according to action on blood agar* may be carried out as follows:

(a) *Streaked Plates*.—With each specimen inoculate 2 plates of 5 per cent defibrinated horse or human blood-agar medium by streaking the surface of each in parallel rows with one loopful of material, undiluted or diluted, depending upon the number and kind of microorganisms found in the preliminary microscopic examination. Incubate one plate under anaerobic conditions in a hydrogen jar and examine for growth after forty-eight hours; incubate the other in an atmosphere which contains from 5 to 10 per cent carbon dioxide and examine after twenty-four hours. If no growth characteristic of the hemolytic streptococci is present, reexamine after longer periods of incubation.

(b) *Pour*
agar which
to 45° C.
the tube. With a pipet place 1 cc. of appropriate dilutions of the specimen—1 in 10, 1 in 100, etc.—in a Petri plate and add the melted blood agar. Tilt the plate carefully to distribute the inoculum evenly. Before the plates are inverted for incubation, be sure that the medium has solidified. After incubation for twenty-four hours, examine for colonies surrounded by clear, colorless zones of hemolysis; if none are found, reexamine after longer incubation.

As a control of aseptic technic and of the sterility of defibrinated blood, prepare at least one uninoculated plate of the blood-agar medium and incubate with the inoculated plates.

(c) *Streaked-poured Plates*.—Inoculate beef-heart-infusion agar plates by streaking the surface as described for streaked plates. Pour over the inoculated surface a thin layer, approximately 5 cc. of 5 per cent defibrinated horse or human blood agar. After the medium has solidified, invert the plates and incubate for twenty-four hours. Examine the plates from the bottom for hemolytic colonies; if none are found, reexamine after further incubation. Similarly, prepare and incubate a sufficient number of uninoculated plates of the blood-agar medium to serve as a control of sterility.

It is frequently necessary to culture blood from conditions in which streptococci are suspected as the etiological agent. In such cases the usual precautions regarding adequate broth-to-blood ratio should be observed as well as the need for the use of para-aminobenzoic acid in the infusion broth medium in which the blood is to be cultured in those cases

where sulfonamides have been used. Some workers prefer a semi-fluid infusion agar for such purposes. Such a medium containing $\frac{1}{8}$ per cent agar is warmed to 35° C. prior to the addition of the patient's blood. Blood clots, aseptically handled, may be comminuted for culture according to the method of Sellers and Morris by placing the clot in a suitably large, sterile syringe and forcing it, with the plunger, through the syringe tip into a flask of broth or a bottle of semi-fluid agar. Cultures must be incubated at 37° C. until growth appears and are not to be considered negative until after at least seven days incubation for plates and fourteen days for flasks and bottles. In the case of endocarditis repeated blood culture may be necessary since the bacteria concerned, streptococci or otherwise, are usually only released from the heart valve lesions into the blood at intervals. Material from broth or semi-fluid agar showing growth should be streaked on blood agar plates to determine hemolytic activity and to obtain colonies for pure culture study.

The fibrinolytic action of streptococci is determined as follows: Dilute 0.2 cc. of oxalated human plasma (0.02 gm. of potassium oxalate to 10 cc. of blood) with 0.8 cc. of saline. Add 0.5 cc. of a young (eighteen to twenty-four-hour) turbid broth culture of the streptococcus to be tested. Mix immediately and add 0.25 cc. of a 0.25 per cent aqueous solution of calcium chloride. Mix and place in a water bath at 37° C. In about ten minutes there should be a solid coagulum. Observe frequently and note the time when the contents of the tube become completely fluid. Plasma from individuals who have recovered recently from hemolytic streptococcus infections, is not suitable for the test.

Fermentation of Carbohydrates.—The data given in the tables in this section indicate the various fermentation tests which may be used in streptococcus study. Routinely these tests are seldom utilized. Of the tests the failure of the streptococci to produce acid from inulin Hiss' serum water is important because pneumococci give a positive reaction in this medium.

3. *Animal Inoculation.*—*Mouse Inoculation.*—Streptococci may be virulent for white mice causing the death of these animals of septicemia but the

mor

they fail to ferment mannitol and D-glucose
pneumococcus.

Virulence Test.—No test is available for evaluating the virulence for man of streptococcus cultures isolated from carriers or convalescents.

4. *Serological.*—The grouping of streptococci is carried out utilizing prepared precipitating sera and extracts of the cultures to be tested. The positive test is a precipitation observed as a ring reaction forming at the junction of antigen and antibody after some ten minutes incubation at 37° C. The ring formation may not appear so promptly and if so the tubes should be shaken and incubated for two hours. At this time they should be examined for precipitation and not recorded as negative until the tubes have been held overnight at from 4° to 6° C. Eleven groups are known but interest centers chiefly on groups A, B and mercially available. The typing of streptococci by the Slide Agglutination Test is carried out with of the streptococci to be tested. Because of

be applied only to streptococci known to be group A. To facilitate results the serums are available individually and in pools. As in pneumococcus typing the culture is first located in one of the pools (usually five) and then by subsequent test identified with one of the components of the positive pool. Typing and grouping are refinements of technic many laboratories cannot carry out and they should never be carried out except with proper materials and by experts. They are however very useful epidemiological tools and every effort should be made to utilize these procedures which experience suggests will be increasingly important.

5. **Confirmatory Test.**—*Hemolysis of Washed Erythrocytes.*—Add 0.5 cc. of a broth culture to an equal volume of a 5 per cent suspension of washed erythrocytes and incubate the mixture in a water bath at 37° C. for two hours. Examine at intervals for hemolysis.

6. **Susceptibility Test.**—*The Dick Test for Susceptibility to Scarlet Fever Erythrogenic Toxin.*—Inject intradermally on the anterior surface of the forearm 0.1 cc. of scarlet fever toxin diluted to contain one Skin Test Dose. This toxin is heat stable and the reaction is read in twenty-four hours so that a control is less generally used than in the somewhat comparable Schick test for susceptibility to the action of diphtheria toxin. The readings are as follows: *Negative*—red area less than 1 cm. in diameter. *Slight positive*—red area 1 to 2 cm. in diameter. *Positive*—red area 2 to 3 cm. in diameter with some swelling and tenderness. *Strong positive*—red area over 3 cm. in diameter, swollen and tender.

NEISSERIA

This genus is defined in Bergey's Manual as follows: "Paired Gram-negative cocci with adjacent sides flattened, which grow as aerobes, facultative anaerobes. Found on mucous membranes, or invading blood stream and localizing in tissues, joints or meninges of mammals. Limited biochemical activity: few carbohydrates utilized, indol not produced, nitrites not produced from nitrates, catalase produced abundantly."

The genus *Neisseria* includes 11 species. Of these the most important pathogens are *N. gonorrhææ* and *N. intracellularis*. *N. catarrhalis* has been found associated with epidemics of conjunctivitis, in upper respiratory infections and in normal individuals. The other species appear to be non-pathogenic parasites of the mouth and upper respiratory tract of man.

Key to the Species of the Genus *Neisseria*

I. Aerobes, facultative anaerobes.

- A. Grow best on special culture media or on plain agar containing blood, blood serum or ascitic fluid, especially with added dextrose. Grow best at 35° to 37° C.; no growth below 25° C. or above 40° C. Acid only in limited sugars. Not chromogenic.

1. Acid from dextrose, not from maltose. Growth can occur anaerobically.

1. *N. gonorrhææ*.

2. Acid from dextrose and maltose. No growth anaerobically.

2. *N. intracellularis*.

B. Grow well on ordinary culture media. Grow well at 22° C.

1. Non-chromogenic.

(a) Moist colonies on agar. No action on dextrose, sucrose or mannitol. 3. *N. catarrhalis*.

(b) Dry crumbly colonies on agar. Acid from dextrose and sucrose; but not from mannitol.

4. *N. sicca*.

2. Chromogenesis—best seen on Loeffler's serum.

(a) Acid from levulose.

(1) Acid from sucrose. 5. *N. perflava*.

(2) No acid from sucrose. 6. *N. flava*.

(b) No acid from levulose.

(1) Acid from dextrose. 7. *N. subflava*.

(2) No acid from dextrose. 8. *N. flavescens*.

II. Anaerobes.

A. Gas produced from peptones.

9. *N. discoides*.

B. No gas produced.

1. Odor of rancid butter.

10. *N. reniformis*.

2. No rancid odor.

11. *N. orbiculata*.

Neisseria Gonorrhœæ

Habitat.—Strict parasite of man. Found in discharges from the genito-urinary system in acute or chronic gonorrhœa, in pus from the conjunctiva in gonorrheal conjunctivitis, and recovered from the blood, joints or even cerebrospinal fluid in those less common cases in which the gonococcus is the cause of endocarditis, arthritis or meningitis.

may develop a roughened surface and a crenated edge.

Blood Agar Plate (Horse). *Twenty-four Hours at 37° C.*—Little or no growth of freshly isolated strains. *Neisseria* are inactive on blood mediums producing neither partial nor complete hemolysis.

Loeffler's Serum. *Twenty-four Hours at 37° C.*—Scanty growth when first

minutes.
may live

s 37° C.

tions. Autolysin present.

Biochemical.—Produces acid, no gas in glucose. No change in litmus milk. Catalase present. Methylene blue reductase absent.

Serological.—Gonococci are immunologically heterogeneous but appear to fall into two main

and ophthalmia neonatorum in man. rabbits, but animals. al transfers. ages, loss of colonies.

Examination of Clinical Materials.—1. **Microscopic.**—Make direct smears of the infected material on glass slides, fix with heat and stain by Gram's stain. To control the staining reaction of such preparations one loopful of a young broth culture of *Staphylococcus aureus* (Gram-positive) and one of *Escherichia coli* (Gram-negative) are placed on the slide before staining. If these control cultures are not available a satisfactory substitute is a small amount of dental "tartar" taken from teeth at the gingival margin with a moistened match end. Such material usually contains a wide variety of microorganisms and may show epithelial and pus cells. The gonococcus is typically Gram-negative when stained properly. It is usually arranged in pairs with flattened, approximating surfaces suggesting a biscuit or coffee-bean shape. The organisms may be observed inside or outside of the pus cells. The intracellular location is typical and has considerable diagnostic significance. Extracellular gonococci, as a rule, are found only in smears from chronic cases of the disease. Smears coming from females

microscopic diagnosis cannot be made. Material for culture must not be allowed to dry and should be plated promptly. If more than a short interval of time is to elapse before it can be cultured it should be kept at from 4° to 10° C. instead of at body temperature. Best results are generally obtained in all culture work, no matter what organism is concerned, when cultures are made immediately after the specimen is collected. Carpenter states that dependable results can be obtained with specimens held for as long as eight hours.

A variety of culture media have been suggested for the growth of the gonococcus from infected material, such as plasma agar, beef or sheep blood agar, starch casein-hydrolysate agar, horse plasma hemoglobin agar and "chocolate" blood agar. Carpenter prefers "chocolate" agar made from an infusion agar base, from a tryptic digest agar base, such as Douglas agar, or from hormone agar base. Human, horse, rabbit or sheep blood may be used to prepare the "chocolate agar" and Carpenter recommends the addition of tyrothricin to yield a final concentration of 1 to 15,000 to inhibit such Gram-positive organisms as streptococci, lacto-bacilli and diphtheroids.

Cultivation is carried out in an atmosphere containing 8 to 10 per cent of carbon dioxide. This requires jars in which the required carbon dioxide concentration can be maintained by displacement of the air present with a prepared mixture of air and carbon dioxide. A simpler method, found very satisfactory, is to place a short-lighted candle on the plates in the

jar and then replace the lid fastening it tightly. When the flame is extinguished by the depletion of oxygen, the concentration of carbon dioxide is approximately 10 per cent. Cultures must be incubated in a moist atmosphere at 37° C. for twenty-four hours but it should be remembered that some strains do better at 35° C. and may require forty-eight hours to grow to recognition. The gonococci appear as convex colonies from 1 to 3 mm. in diameter. Diagnostic features are the undulate character of the colony margins and the transparency of the growth. These features permit distinction from young colonies of streptococci or diphtheroids for which the gonococcus colony may be mistaken.

When uncertainty exists regarding the presence of gonococcus colonies resort may be had to the "oxidase test." This test is based upon the production of an enzyme, oxidase, by organisms belonging to the genus *Neisseria*. Para-aminodimethylaniline monohydrochloride is added to the plate and produces an immediate series of color changes in a gonococcus colony: first pink, then maroon and finally black. If sub-culture is to be made when this dye compound is used it must be done at the "pink" stage because the gonococcus is killed by the test. Its staining reactions are not, however, interfered with. The test is carried out by dropping 1 to 2 cc. of the chemical in solution on the plate to be tested and tilting the plate so that the entire surface is moistened. A "nasal" atomizer can be used to supply the hydrochloride if many plates are to be examined. Some workers prefer tetramethylparaphenylenediamine hydrochloride as the dye to use in this test. The positive test is given by a variety of bacteria other than the gonococcus but most of these, such as species of *Hemophilus*, yeasts, coliform bacteria, *Bacillus* and *Streptothrix* can be ruled out by morphology. Others, such as other species of *Neisseria* can be eliminated by fermentation tests.

Fermentation Reactions.—Fermentation reactions may be determined for pure cultures of *Neisseria* by inoculating pure cultures of the organism to be tested into serum-water media containing dextrose, maltose, sucrose and levulose respectively. More recently the medium preferred for fermentation work has been ascitic fluid agar with Andrade's indicator to which 1 per cent of the fermentable substance is added and which is dispensed in slants in rubber stoppered tubes which effects a limitation of oxygen supply and an increase in carbon dioxide content as growth progresses. Semi-solid mediums may also be used with success. It is advised to observe fermentation studies on *Neisseria* during the progress of the incubation since in some cases acid production is transient. This is particularly so with freshly isolated strains.

Neisseria of medical significance will not ferment lactose. Other fermentations and reactions helpful to differential diagnosis are summarized in Table 63.

3. Serological.—The complement-fixation test is sometimes utilized when a gonococcus infection is suspected and infective material is not available for microscopic and cultural study. Such a test will obviously only succeed when the patient's blood contains the specific antibodies and the antigen used is satisfactory. The test thus has usefulness only in disease of long standing such as arthritis and endocarditis and the antigen purchased or made must be a polyvalent one. Detailed instructions for the complement-fixation test may be consulted in Diagnostic Procedures and Reagents.

of the genus *Neisseria* that produces such translucent colonies is the gonococcus and its colony is usually smaller and there is less tendency to confluence.

Branham states that it is becoming more and more common to make blood cultures as an aid to diagnosis in meningococcic infections. This procedure is of value in early cases and in those without meningeal symptoms.

Fermentation Reactions.—In identifying an organism as a meningococcus resort may be had to fermentation reactions as indicated elsewhere in this section.

3. *Animal Inoculation.*—Test not used. Like the gonococcus meningococci are toxic for animals in sufficient dose but they do not produce characteristic disease in laboratory animals which can be used either to identify the organism or comment on its virulence for humans. Meningococci are known to be present in the throats of the healthy to a certain degree most of the time. It is only when the number of such carriers rises above a threshold figure and when the host population becomes susceptible that more than sporadic cases of meningitis appear. As in pneumococcus work, it would be highly desirable if there were an animal test which would reveal the relative virulence for humans of different strains under study.

4. *Serological.*—(a) *Macroscopic Tube-Agglutination Test.*—The identification of cultures thought to be meningococci is carried out by agglutination tests using suspensions of twenty-four hour growth on solid medium prepared con- the suspending erum dilutions in series from 1 to 25 to 1 to 1600 or higher are prepared. One-half cc. of bacterial suspension is added to each tube (0.5 cc. diluted serum) giving a total volume of 1 cc. with dilutions from 1 to 50 to 1 to 3200. Controls consist of bacterial suspension in saline only and in horse serum 1 to 25 and 1 to 50. The tubes are incubated at 56° C. for eighteen hours and then read.

(b) *Microscopic Slide-Agglutination Test.*—The advantage of this test is that individual colonies from a primary culture may be used and a presumptive diagnosis arrived at quickly. The method follows: Polyvalent antimeningococcus horse serum and normal horse serum are diluted 1 to 10 with 0.85 per cent saline and a loopful of each placed on a slide. Some of the suspected colony is rubbed up in each. A drop of saline is also placed on the slide and receives a charge of the suspected meningococcus culture. If agglutination takes place in the drop containing antimeningococcus serum but not in the normal serum and the saline controls, a presumptive diagnosis of "meningococcus, type undetermined" may be made. Any other reaction is unsatisfactory and should be disregarded. Strains of meningococci may be encountered which fail on occasion to agglutinate in polyvalent antimeningococcus horse serum. Branham states that as a

Noble Method.—A recent development is the production by Phair and associates of highly potent and specific chicken serum for the typing of meningococci by an

Arlyle Noble agglutination technic. The technic consists in mixing in a series of small tubes 0.1 cc. of a heavy suspension (matching the No. 8 tube of the McFarland nephelometer) of the organism with a similar volume of a chicken, type specific, antimeningococcus serum, diluted with saline 1 to 1, 1 to 2, 1 to 4, 1 to 8, 1 to 16 and 1 to 32. The mixtures are agitated for three minutes and then to each tube 0.8 cc. of saline are added. The resulting reactions may be classified almost immediately as complete, almost complete, partial, slight or no agglutination and recorded as 4 +, 3 +, 2 +, 1 + or 0, respectively. This reaction takes place at room temperature, is read immediately, and the agglutinated organisms form clumps which can be seen easily. It is hoped that this method will provide a better means of expl . . . of the meningococci

(d) *Identific* . . . —The demonstration of capsular swelling when an organism is brought into contact with its specific antiserum, a reaction of first importance in dealing with pneumococci, has been suggested for the meningococci found in cerebrospinal fluid by Clapp and studied by Einhorn. It is possible that meningococci may be dealt with by this technic but their rating as "non-encapsulated" makes it unlikely that the test will ever establish itself as of much value. It has long been known that a certain "volumination" results when organisms are brought into contact with specific antiserum. The difficulty is demonstrating such a reaction in the absence of distinct capsular material makes such methods difficult and unsatisfactory for general use. (Type 2 [alpha] lends itself to this technic better than other types)

(e) *The "Halo Reaction."*—Of much more significance for practical purposes is Petrie's "Halo Reaction," originally described for several species of organisms but only applied in practice to the meningococcus. The reaction is the development of halos around colonies of meningococci on agar plates containing immune serum and is due to the precipitate formed by the interactions of the soluble specific antigens of the meningococci and the specific precipitins in the serum.

The following directions for carrying out the "Halo Reaction" are approximately those of Branham:

1. Melt the agar in tubes, each tube containing approximately 15 to 16 cc.

2. Cool the agar to 50° C., add 0.8 cc. of serum and pour the mixture into sterile Petri dishes. This gives a serum concentration of approximately 5 per cent.

3. Collect a mass of growth with a platinum loop, about 2 mm. in diameter, from an eighteen-hour culture on glucose serum agar or five-hour culture on blood agar.

4. Place this mass upon the surface of the specific serum agar plate without spreading; 6 to 10 different cultures may be tested on one Petri dish.

5. Incubate the plate at 37° C., and examine it after forty-eight and seventy-two hours in a strong light against a dark background. A visible precipitate in the form of a halo around the bacterial growth will be noted in a positive reaction. Variations of intensity of halo formation may be recorded as 1 plus to 4 plus.

Neisseria Catarrhalis

Neisseria catarrhalis is a non-motile, Gram-negative diplococcus, at times arranged in tetrads or small groups. In sputum the organisms are shaped like coffee-beans, and may be both intra- and extracellular. In culture they are generally larger, growing in tetrads and staining evenly. On agar, after twenty-four hours, the colonies are about 1 to 2 mm. in diameter, convex, whitish-gray, with a glistening surface and an entire edge. After three or four days they are 3 to 4 mm. in diameter, and are differentiated into a prominent, more elevated, opaque, slightly brownish center and a thinner, gray, transparent, wave-like periphery with a crenated edge. The colonial appearance is subject to variation, both "smooth" and "rough" types being formed, but they are generally coherent, tenacious, membranous, friable, difficult to emulsify, and auto-agglutinable when suspended in saline. When grown in broth there is usually only slight turbidity, the sediment is coarsely granular and does not disintegrate completely on shaking. If the tube is not shaken a pellicle may appear. Growth occurs within a range of 18° to 42° C., the optimum being at 37° C. The organism is aerobic and will not grow under strictly anaerobic conditions. No sugars are fermented. Gelatin is not liquefied. Growth is favored by the addition of blood, serum and ascitic fluid, but not by glycerol. Cultures in a moist state will survive for four or five months at 21° C. The organisms live in dried sputum for as long as twenty-seven days. They are killed by heating to 65° C. for thirty minutes. The virulence for laboratory animals is low. Rabbits are resistant but guinea pigs injected with large doses die of toxemia in twenty-four hours. Heat-killed cultures are almost as fatal as living ones. Diagnosis is made from the infected secretions by microscopic examination (the organisms are larger than meningococci and may not be arranged in pairs) and by culture. Growth at room temperature and on simple media will exclude the gonococcus and the meningococcus. None of the sugars are fermented.

STAPHYLOCOCCI AND RELATED MICROCOCCI

Staphylococcus

This genus is defined as follows: "usually parasitic, cells occur singly, in pairs and in irregular groups, rarely in packets. Usually Gram-positive. Growth fair to good on the surface of artificial media. As a rule carbohydrates are fermented with the formation of acid. Gelatin commonly liquefied. Nitrate reduction variable. May produce hemolysis on blood agar. Pigment white or orange, or less commonly lemon yellow."

Nine species are listed in Bergey's Manual (1939). Of these *Staphylococcus aureus*, *Staph. citreus*, *Staph. epidermidis*, *Staph. albus*, *Staph. muscae* and *Staph. pharyngis* are aerobes; and *Staph. asaccharolyticus*, *Staph. aerogenes* and *Staph. anaerobius* are anaerobes. *Staph. aureus* produces a

Staph. albus and *Staph. muscae* ferment sucrose and mannitol but not raffinose. *Staph. albus* is found on skin and mucous membranes whereas *Staph. muscae* causes a fatal infection in house flies transmissible to healthy

flies. *Staph. pharyngis* ferments sucrose, mannitol and raffinose. The six aerobic species typically liquefy gelatin and ferment lactose. The anaerobic staphylococci produce gas from peptones in some cases with a fetid odor and are found in body cavities. *Staph. xrogenes* has been found in cases of puerperal fever and recovered from infected tonsils. The anaerobic species do not liquefy gelatin or ferment lactose. The type species is *Staph. aureus*.

Staphylococcus Aureus

Habitat.—The skin and mucous membranes. The cause of boils, carbuncles, abscesses, suppurating wounds, osteomyelitis, occasionally meningitis and septi-

cally, in pairs, or short chains; non-motile; non-flagellated; non-capsulated and non-sporulating. Stain easily with aniline dyes; Gram-positive and non-acid-fast.

Resistance.—Withstands moist heat at 60° C. for thirty minutes, usually killed in one hour. May stand drying for months. Killed in fifteen minutes by 2 per cent phenol. Inhibited by basic triphenylmethane dyes. One of the most resistant of

in diameter in twenty-four to forty-eight hours at 37° C.

Blood Agar.—Abundant growth. Clear zone of beta hemolysis around golden yellow colonies.

Loeffler's Coagulated Serum.—Abundant raised confluent golden yellow growth.

Eosin-Methylene Blue Agar.—Growth inhibited.

Nutrient Broth.—Moderate uniform turbidity; powdery deposit which disintegrates readily on shaking; slight ring of growth at surface.

Gelatin Stab.—Saccharose zone of liquefaction with yellowish pellicle and yellow to orange sediment.

Litmus Milk.—Acid, coagulated.

Biochemical Reaction

Lactose, glycerol and starch not hydrolyzed

duced. Milk usually coagulated by the acid produced by fermentation, but the precipitated casein usually is not dissolved. Indol not produced. Nitrate reduction variable. Methylene blue reductase positive; H₂S slight; NH₃ produced. Golden yellow pigment formed most readily at 22° C. on albuminous media exposed to

Pathology.—Agglutination and precipitation tests have been used in numerous attempts to classify staphylococci. Precipitation tests with specific carbohydrates extracted from the organisms have been advocated to separate staphylococci into types A and B respectively. Most of the type A strains ferment mannitol and those of type B do not. Serological tests are rarely used in routine diagnosis.

Pathogenicity.—Most virulent cultures are those recently isolated from human lesions. Man more susceptible than laboratory animals. Pure cultures rubbed into unoperated skin of man have produced carbuncles. Probably the cocci penetrate through sweat ducts or at base of hair follicles. Natural infections common, resulting in severe pyogenic infections (boils, carbuncles, abscesses, osteomyelitis, septicaemia, pneumonia). Staphylococci have also been demonstrated as causing an

important type of "food-poisoning." Experimentally pathogenic for rabbits, less so for mice and guinea pigs; rats and pigeons are said to be resistant.

Dissociation.—Variations which may occur include changes in morphological, . . . Newly isolated pathogenic
when held in the laboratory.

... says: "The general opinion is
c,
g-
"

Staphylococcus Albus

Staphylococcus albus differs from *Staph. aureus* in color, being white instead of golden yellow and usually much less pathogenic and biochemically active. Otherwise they are similar.

Staphylococcus Epidermidis

Staphylococcus epidermidis is another organism which produces white colonies and may give rise to minor lesions such as stitch abscesses. It is probably only a variety of *Staph. albus*.

Staphylococcus Citreus

Staphylococcus citreus also resembles *Staph. aureus* in many respects, but it is a non-pathogenic saprophyte which produces a lemon-yellow pigment.

GAFFKYA

This genus includes *G. tetragena* and three other species. They differ from organisms of the genus *Staphylococcus* in that they occur as tetrads in the animal body and in special media, while in ordinary culture media they occur in pairs and irregular masses. They also differ in other details such as the failure of the more important species to liquefy gelatin.

Gaffkya Tetragena

G. tetragena is a Gram-positive parasitic coccus; found in the mucous membranes of the respiratory tract; isolated from pulmonary cavities in tuberculosis and from abscesses in man and lower animals. It has also been found in air and on the normal skin. In pathologic materials it occurs in tetrad arrangement and may be surrounded by a thick halo-like capsule. It grows on the usual culture media, often producing more

The organism is feebly pathogenic for guinea pigs and white mice.

SARCINA

This genus is defined as follows: "Saprophytes and facultative parasites. Division occurs, under favorable conditions, in three planes, producing regular packets. Usually Gram-positive. Growth on agar abundant, usually with formation of yellow or orange pigment. Dextrose broth

slightly acid, lactose broth generally neutral. Gelatin frequently liquefied. Nitrates may or may not be produced from nitrates." In Bergey's Manual (1939) 14 species are identified including *Sarcina lutea* which is found in air, soil, water and on skin surfaces and *Sar. ventriculi*, a characteristic organism from the normal stomach contents of man.

Examination of Clinical Materials for Staphylococci, Gaffkya and Sarcina.—The organisms here considered together—Gram-positive cocci, growing readily on most media, and found normally in the air and on the skin as well as in occasional disease conditions may be present in infected material from abscesses, carbuncles, boils, pustules, osteomyelitis and occasionally in septicemia and even pneumonia and meningitis. Since some of these bacteria, especially *Staph. albus*, are found on the normal skin and mucous membranes their presence in a lesion does not necessarily incriminate them as the etiological agent for they are common contaminants in cultures from open lesions.

1. **Microscopic.**—Examine thin films stained by Gram's method for morphologically typical organisms. Under favorable conditions *Gaffkya tetragena* will show encapsulated tetrads and *Sarcina lutea* will appear in twos, fours and cubical packets of eight. Mostly, the staphylococci and related micrococci however, will be arranged as single cells, pairs, or in groups containing irregular numbers of individuals. They are less apt to be found intracellularly in the presence of leucocytes than are gonococci.

2. **Cultural.**—Inoculate nutrient agar or blood agar plates with the purulent material or in suspected cases of bacteremia make cultures of the blood in the usual way. Growth on the plates will be abundant after twenty-four hours incubation at 37° C. Pigment may be confirmed by spreading a loopful of growth on white paper and noting the color of the dried organisms or by inoculating suitable media and incubating at room temperature. Confirmation of the results obtained by plating or blood culture may be made by microscopic examination and by cultural studies of sub-cultures. There is no cultural test which has proved entirely satisfactory for use in determining the enterotoxin production of *Staph. aureus*, a very important matter in view of the frequency with which staphylococcus food poisoning occurs and the desirability for determining the source of such outbreaks.

3. **Animal Inoculations.**—Animal inoculation may be undertaken with cultures of staphylococci suspected of causing food poisoning. The animal of first choice for the demonstration of enterotoxin is, according to Hopkins and Poland, the suckling pig. Since this test animal may not be easy to procure the improved cat test of Hammon may be described. This test consists in the intravenous injection into a healthy adult cat of 0.5 cc. to 5.0 cc. of the filtrate presumed to contain the staphylococcus toxin. This toxin should previously be heated in a boiling water bath for thirty minutes to destroy the lethal factor or factors it may contain. The same cat may be used for two or even three tests but at each succeeding test a larger volume of suspected toxin must be given. One should not accept as final the result of a negative test unless repeated on at least two previously unused animals in a minimum dose of 3 cc. Vomiting is the specific positive reaction in the cat which intravenous injection of staphylococcus enterotoxin calls forth. There will usually be other signs and symptoms but vomiting only can be accepted as specific. If the cat has been offered

and takes a moderate meal before the test is done the effectiveness of the vomiting stimulus is increased and the fact that the cat ate readily is an indication of its health. Feline epizootic panleucopenia is a common infectious disease of cats, producing vomiting and diarrhea hence it is quite important that the cats used be healthy.

4. *Serological*.—Extensive immunological studies have been carried out on staphylococci but there are no groupings or typings or other identifications to be made by such methods for staphylococci and related micrococci at the present time.

5. *Confirmatory Tests*.—*Coagulase Test*.—A fully virulent freshly isolated staphylococcus has several characteristics such as golden-brown pigmentation, hemolytic activity and the ability to readily liquefy gelatin and ferment mannitol. The production of coagulase, is, however, reckoned by many workers as the most important single biological characteristic of a virulent staphylococcus. The coagulase test is carried out as follows:

Diluted plasma (1 part plasma, 4 parts normal saline) is distributed in 0.5 cc. quantity in a small tube for each test to be made. One-half cc. of a twenty-four hour broth culture or a small loopful of growth obtained from agar is added to and mixed with the diluted plasma. The tube, or tubes, is placed in the 37° C. water bath and examined at fifteen to thirty-minute intervals for evidence of clotting. A clot usually forms within two hours if the organism added is coagulase positive. Plasma for use in this test may be obtained either from human or rabbit source and may be preserved in the lyophilized state.

VIBRIO

Organisms of the genus *Vibrio* are described thus: "Cells short, bent rods, rigid, single or united into spirals. Motile by means of a single (or rarely two or three) polar flagellum, which is usually relatively short. Many species liquefy gelatin and are active ammonifiers. Aerobic, facultative anaerobic. No endospores formed. Usually Gram-negative. Water forms; a few are parasites."

The genus *Vibrio* contains 21 acceptably described species and Bergey further lists 33 inadequately described species which have been mentioned in the literature. In addition to occurring as harmless saprophytes in water, soil, cheese, hay infusion and rotted stable manure certain species of the genus are found in abortion in cattle, insect diseases, chronic endometritis, infections of fish and frogs, sputum, and fatal infections of fowls and birds. It must again be emphasized that a distinction is to be made between "being found in a disease condition" and being the etiological agent of a disease. The genus *Vibrio*, however, does contribute one species (the type species), *viz.*, *V. comma*, of undoubted and dramatic etiological significance as the cause of Asiatic cholera.

Vibrio Comma

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isms may be small, resembling granules, and stain poorly. Involution forms numer-

low convex, translucent, grayish-yellow, surface, and an entire edge. They are but easily emulsifiable.

gle polar flagellum. Gram-negative, non-

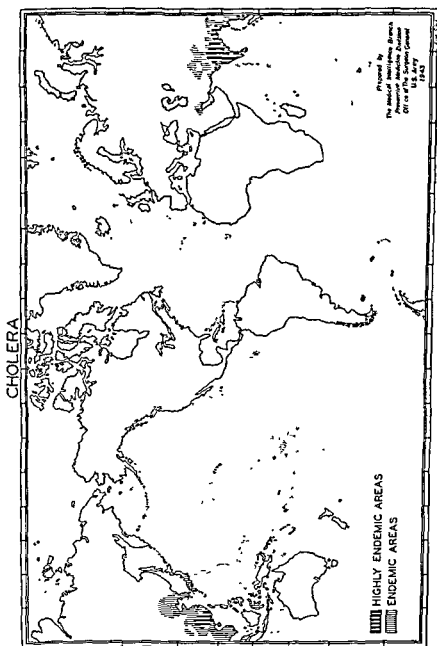


FIG. 38.—World distribution of cholera

round, grayish, transparent colonies.

Hours at 37° C.—Abundant growth. zone of alpha or beta hemolysis.

four Hours at 37° C.—Large, smooth,

growth, confluent at top, uniform or napiform lique-

Loeffler's Serum. Ten Days at 37° C.—Good growth with partial liquefaction.

Potato. Seven Days at 37° C.—Good confluent, brownish growth with glistening surface.

ature 37° C, limits 16° to

6.4 to 9.6. Growth favored

peptone water. No soluble

hemolysin formed for sheep or goat cells. Proteolytic and diastatic ferments

secreted.

acid at bottom, not coagulated, but is slowly peptonized. Indol +; cholera red

reaction +; M. R. -; V. P. -; nitrates reduced; NH₄ +, H₂S + in fourteen days;

catalase +; M. B. reductase +.

Serological.—Strains of cholera and cholera-like vibrios which possess general

chemical similarity, have, in the smooth state, a common H antigen. The O antigen

exists in different immunological form in different strains at least six such groups

by the Pfeiffer test; also precipitins and agglutinins.

Pathogenicity.—

Examination of Clinical Materials.—*Vibrio comma* may be isolated from

the stools of cases or carriers and at times from contaminated water or

foods. The feces may be collected as for other bacteriological examinations,

selecting if possible, in the case of a patient, a portion of the "rice-water"

stool. In the case of a carrier survey of healthy persons specimens may

have to be obtained through the use of saline cathartics, particularly when

it is necessary to detain a ship or a caravan coming from an area where

cholera is occurring. No glycerol or other preservative is added to the

fecal specimen. Where water is to be examined collect 1 liter of surface

water in a sterile flask.

1. *Microscopic.*—The microscopic examination of the stool may be of

great value in making a presumptive diagnosis in suspected cases, but is

of no use in the examination of carriers. Smears are made of a flake of

mucus from the stools, which in a typical case, has the well-known "rice-

water" character. Stain by Gram's method and with dilute carbolfuchsin. If typical Gram-negative, comma-shaped organisms are present, examine a hanging-drop preparation. If large numbers of typical, actively motile vibrios are found, a preliminary report may be made. In preparations from intestinal contents the vibrios are often "arranged like fish in a stream." However, this tentative diagnosis must be confirmed by cultural and serological examinations.

2. **Cultural.**—(a) *Feces.*—Specimens of feces from suspected cases or carriers should be planted, using two or more loopfuls of intestinal mucus or liquid feces with the least possible delay and incubated at 37° C. Use:

(1) Alkaline peptone water, pH 8.0 to 8.4.

(2) Alkaline nutrient agar, pH 8.0 to 8.4, or

(3) Dieudonne's agar.

After six to eight hours at 37° C., examine hanging-drop and stained film preparations made from the surface growth of peptone water. Presumptive diagnosis is obtained if the preparations show actively motile, Gram-negative vibrios in large numbers. Further confirmation by culture and serology must be made.

(b) *Water.*—Water under test is placed in 100-cc. amounts in sterile flasks to each of which are added 10 cc. of 10 per cent peptone water. After six to twelve hours incubation at 37° C. a portion of the surface growth is transferred to fresh tubes of alkaline peptone water and plated out on alkaline nutrient agar or to Dieudonne's agar.

Linton states that the main emphasis in recent cholera research has been to differentiate between pathogenic and non-pathogenic forms. He would accept as an authentic cholera organism a Gram-negative, actively motile vibrio which fermented mannose and sucrose but not arabinose, which failed to hemolyze goat's red blood cells and which agglutinated with O-group I serum. While the demonstration of gelatin liquefaction and a positive "cholera-red" reaction are confirmative they are not specific. A hemolytic strain giving the above reaction would throw a culture into the disputedly pathogenic "El Tor" group.

Cultural confirmation, therefore, consists in obtaining a pure culture from presumptive, or subsequent plates, and planting it on a slant from which material may be taken for the fermentation and hemolysis tests indicated and for serological confirmation.

3. **Serological.**—(a) *Presumptive Test.*—Deposit, near one end of a slide, a drop of agglutinating serum of a dilution of 1 to 200 (titre not less than 1 to 1000) and near the other end a drop of saline; also place a third drop consisting of normal serum (diluted 1 to 10) near center of the slide as a control. Then touch the suspected surface growth with point of the inoculating needle and rub up in the drop of saline solution; flame the point, again touch the surface pellicle with the point and rub it in the drop of serum dilution; flame the point of the platinum needle again and add bacteria to the serum control in the same manner. Agglutination will almost instantly appear in the anti-cholera serum (if cholera). The drops may be allowed to dry; then fix and stain; if agglutination has taken place, it will be evident, in the stained specimen, to the naked eye or on slight magnification with the hand lens.

(b) *Confirmatory Test.*—A suspected *Vibrio* culture may be confirmed serologically by macroscopic tube-agglutination methods using as

antibody an "O-group I" serum in indicated dilution. A common "H" antigen is found for all vibrios biochemically similar to the true *Vibrio comma*. This is heat-labile whereas the six (or more) "O" antigens are heat-stable. Agglutinating sera are, therefore, prepared against heated strains of the respective group types.

4. *Animal Inoculation.*—*Vibrio comma* produces a fatal peritonitis within twenty-four hours when a loopful of living culture emulsified in 1 cc. of broth is injected intraperitoneally into a guinea pig. It produces only slight reaction when injected intramuscularly into a pigeon. On the other hand *V. metchnikovi* is rapidly fatal for both pigeons and guinea pigs. Although non-pathogenic for man this vibrio is far more invasive for animals than is *V. comma* and, since it closely resembles Koch's "commabacillus" biochemically, situations might arise in which the pigeon test could be most helpful. *V. proteus*, *V. tyrogenus* and *V. strictus* are species which have some guinea pig pathogenicity but they differ biochemically from *V. comma* to such an extent (dextrose and sucrose negative, indol negative) that confusion should not occur besides which only *V. proteus* is found in the intestine.

Pfeiffer's reaction, the demonstration of *in vitro* bacteriolysis, carried out by injecting cholera vibrios and specific antiserum into the peritoneal cavity of a guinea pig and observing the resultant peritoneal exudate for the destruction of the injected organisms, is a reaction of great historical interest, but of only incidental practical value.

PSEUDOMONAS

The organisms of this genus are defined as "principally water and soil bacteria producing a water-soluble pigment which diffuses through the medium as green, blue or yellowish-green. Motile or non-motile. Gram-negative."

There are 31 species of which the only member of much importance to human medicine is *Pseudomonas aeruginosa*, also known as *Bacillus pyocyaneus*. In addition to its occasional presence in suppuration, the pus of which in contact with the surface dressings assumes a "blue-green" color, the organism, or its relatives, may occasion trouble in technical procedures through persistence in growing out in mixtures inoculated on plates or in tubes often effecting the masking of the organism really sought. Careful plating and colony picking and the incorporation of lithium chloride or potassium tellurite in a medium are aids in handling such a problem. *Ps. aeruginosa* is also frequently found in diarrhea and general infections in infants.

Pseudomonas Aeruginosa

Habitat.—Widely distributed in nature, frequently found on the healthy skin of man, in the feces of many animals, in water contaminated by animal or human material, in purulent discharges and in serous wound secretions.

Morphology.—Rods 0.5 to 0.6 μ wide by 1.5 to 3.0 μ long, axis straight, ends rounded, sides parallel, occurring singly, in pairs or in short chains. Motile, pos-

moist agar surface must also be emphasized.

—Moderate filiform growth to the bottom which may be rapid or slow, the fluid color. Later, the upper 1 to 2 cm. are turbid, yellowish-green, and may occur around the filiform growth.

for production of fluorescent pigment.

Biochemical.—Acid, no gas in glucose. Indol — as a rule, occasionally +. M. R. —; V. P. —; nitrate reduction —; H_2S +; NH_3 production +; catalase +; M. B. reduced; starch diastase —. Litmus milk may show a slight preliminary clot, but shows complete peptonization and decolorization in five days at $30^\circ C$, the milk often turning green.

Serology.—Unexplored.

general
lives rise

Examination of Clinical Materials.—Follow the procedure outlined under Staphylococci for the isolation of *Pseudomonas aeruginosa* from infected material and identify the organism according to the reactions indicated above.

PASTEURELLA

The genus *Pasteurella* is described as "small, Gram-negative, ovoid to elongated rods showing bipolar staining by special methods; aerobic, facultative; require low oxidation-reduction potential on primary isolation; powers of carbohydrate fermentation slight; no lactose fermentation; no gas production; gelatin not liquefied; milk not coagulated; parasitic on man, other mammals and birds."

Key to the Species of Genus *Pasteurella*

1. Growth on ordinary media; growth in milk.

A. Non-motile and non-flagellated at 18° to $26^\circ C$. No change or slight acid in milk without clot.

1. Indol and H_2S produced. No growth in bile. Sorbitol fermented.

(1) Disease in birds and fowls — *Pasteurella avicida*.

(2) Disease in rats and mice — *Pasteurella muricida*.

(3) Disease in rabbits — *Pasteurella cuniculicida*.

(4) Disease in swine — *Pasteurella suilla*.

(5) Disease in cattle and deer — *Pasteurella bovinorum*.

2. Neither indol nor H_2S produced. Growth in bile. Sorbitol not fermented.

(6) Disease in rodents and man — *Pasteurella pestis*

B. Motile and flagellated at 18° to 26° C. Milk alkaline. H₂S produced. Indol not produced.

(7) Disease in rabbits and rats—*Pasteurella pseudotuberculosis*.

II. No growth on ordinary media; no growth in milk.

(8) Cause of Tularemia—*Pasteurella tularensis*.

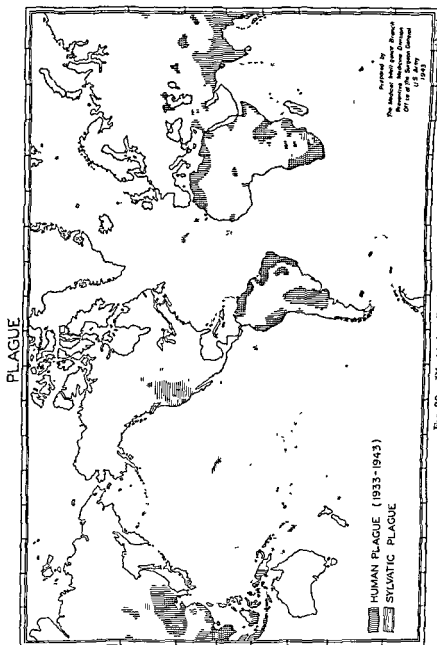


Fig 39.—World distribution of plague.

The genus *Pasteurella* is one of the few in which all described species are pathogenic. From the standpoint of veterinary medicine this is one of the most important groups of bacteria and the two species causing human disease are not without considerable significance. It is common to char-

acterize the *Pasteurella* as "the bipolar-staining organisms of the well known hemorrhagic septicemia group."

Pasteurella Pestis

Habitat.—Primarily a parasite of rats; also other rodents, especially the marmot

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colorless, finely granular, umbonate colonies, with an entire or slightly undulate edge. After five days incubation at 37° C. colonies are raised, nearly opaque, translucent, grayish-white periphery emulsified.

Blood Agar Plate (Horse). Forty-eight Hours at 37° C.—Colonies similar to those on agar, but show less tendency to differentiation and peripheral spread. No hemolysis.

Glycerol Agar.—Growth rapid, forming grayish-white colonies.

Gelatin Stab Seven Days at 37° C.—Good filiform growth, confluent at top, sending out little 5 mm. in diameter,

sep
in 1

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ten

to 8.2. No hemolysin produced.

is infectious for white
and monkeys. Large
sparrows, are resistant.
re is a local edema fol-
nds, and a generalized
larged and surrounded

by hemorrhagic exudate, congestion of spleen and liver which may show small grayish necrotic areas; bacilli are found in the local lesions, bubo, internal organs, especially the spleen, and blood.

for rats.

Transmission.—Plague is transmitted to man primarily by the bites of infected rat fleas, *Xenopsylla cheopis*, *Ceratophyllus fasciatus*, etc. However, the organism may also gain entrance into the body by contact with the abraded skin, through wounds or abrasions, and the respiratory tract.

I. Examination of Clinical Materials.—Evidence of infection with the plague bacillus in man or in rodents may be demonstrated by microscopic, cultural and serological examination of pathological materials and by inoculation of those materials into suitable test animals.

1. Collection of Specimens.—(a) *From Patients.*—Early in the disease a small vesicle may be found at the point of the infective flea bite and plague bacilli may be demonstrated in the fluid from such a vesicle. The bubo which usually appears in the inguinal region should be aspirated and the fluid or pus examined for plague bacilli. In mild or chronic cases the bubo may be hard and difficult to aspirate and in such cases may be excised. At times the organisms occur in the blood but the period of septicemia varies. The plague bacilli are not found in human feces and only occasionally in the urine. In the dangerous pneumonic form of plague the sputum should be examined.

(b) *From Cadavers.*—Collect portions of bubo, spleen and lung and make microscopic, cultural and guinea pig inoculation tests. In frozen cadavers, plague bacilli have been isolated from buboes up to one hundred and two days.

(c) *Rodents.*—The entire carcass is used for examination. If infected animals or other plague materials are to be sent to a distant laboratory the specimens must be placed without any preservative in a tightly sealed container (fruit preserving jar, for instance), which is packed in a second container to avoid breakage and escape of the content. The package should be marked: "*Perishable—For Bacteriological Examination. Please Expedite*" and must be shipped by express as Federal laws prohibit the shipment of plague-infected materials by mail. Decomposition may be avoided by surrounding the specimen container with ice or solid CO₂ (dry ice).

2. Microscopic.—Make smear preparations from the lesions or internal organs (spleen, particularly) and stain by Gram's and Wright's methods and with methylene blue or dilute fuchsin. Wayson's stain, which is a phenol fuchsin-methylene blue in alcohol and water, is particularly recommended for demonstration of bipolar morphology. If typical Gram-negative, short ovoid polar-staining bacilli are found, many of which are degenerated or poorly stained, these may be considered as suggestive, but not conclusive evidence of infection with *Past. pestis*. Rodents, particularly, may be infected with related *Pasteurella* which are morphologically similar to *Past. pestis*; hence the finding of characteristic bacilli must be confirmed by cultural, serological and animal inoculation tests.

3. Cultural.—Inoculate the surface of blood agar, glycerol agar and 3 per cent sodium chloride agar plates with a portion of the suspected specimen. Blood should be cultured in broth before plating, however in the terminal stages of both the bubonic and pneumonic types, the bacilli often are present in large numbers in the blood. After incubation at

4. **Serological.**—(a) *Precipitin Test.*—This test is sometimes used in making a rapid presumptive diagnosis on decayed tissues. One part of the finely divided tissue is mixed with 5 to 10 parts of distilled water, boiled for five minutes and filtered repeatedly through paper or asbestos wool under pressure until clear. Test this antigen with specific immune serum in a small tube. A positive reaction shows a precipitate in five minutes at 37° C., increasing to a maximum after two hours. A negative reaction may not be conclusive.

(b) *Agglutination Test.*—*Past. pestis* in salt solution has a tendency to agglutinate spontaneously, hence the macroscopic method of agglutination is preferred to a microscopic slide method. The test is of greatest value in identifying suspect cultures, positive titre being interpreted in comparison with the titre of the same serum tested with a known plague antigen. The test is of little value as applied to the serum of a patient for agglutinins do not appear in plague patients until about the ninth day of illness. To carry out the test prepare a suspension of a young agar culture in normal saline, allow to stand for a few minutes then use only the evenly turbid supernatant portion of the suspension. High-titre agglutinating antisera, prepared in horses, are generally used to identify the cultures. A series of tubes is set up of antibody dilutions to each of which the antigen is added. Salt solution and normal serum controls should be included.

5. **Animal Inoculation.**—Animals should be freed of all ecto-parasites, prior to use, by dipping in an antiseptic solution. They are then placed in glass jars covered with fine mesh gauze to prevent access or escape of any infected or potentially infected insect vectors. Further precaution directed toward the same end is to grease the upper inner surface of the jar. When handling animals, living or dead, in plague work, protect the hands and arms by wearing rubber gloves and long-sleeved gown.

Inoculate guinea pigs or mice subcutaneously with a small amount of the original specimen, or with a loopful of suspected culture. If *Past. pestis* is present, the animals will develop characteristic lesions and die in two to five days with characteristic postmortem appearance. Cultures of the plague bacillus may be isolated from the lesions and from bone-marrow.

Specimens which have undergone putrefaction may be applied to the freshly shaven abdomen of a guinea pig. The plague bacillus (also *Past. tularensis*) can penetrate the abraded skin whereas contaminants cannot.

II. **Diagnosis of Plague in Rodents.**—The diagnosis of natural infection in rodents is a very important aspect of public health bacteriology. It may be made macroscopically, although occasionally the disease may occur without recognizable lesions. The presence of a bubo is probably the most important single indication. In the early stage the gland is enlarged, congested, and on section shows hemorrhagic points. When the disease is fully developed the gland contains an area of gray necrosis which may or may not be confined to the medulla. The liver is mottled, red and yellow, with small punctate hemorrhages and pinpoint grayish or yellowish spots due to fatty necrosis. The spleen may be enlarged, is firm, and may show small discrete or confluent granules on the surface. Pleural and peritoneal surfaces are infected and the cavities contain an excess of fluid. The most important diagnostic signs of plague are: (1) bubo; (2) subcutaneous and general congestion; (3) granular liver; (4) congested spleen; and (5) pleural effusion. Microscopically, bacilli are found in the bubo,

spleen and blood. Cultures may be made from these sources and studied by the methods outlined above under examination of clinical materials.

It must be remembered that rodents are also susceptible to several other natural infections which may be confused with plague. Among these diseases are those caused by *Past. muricida*, *Past. pseudotuberculosis*, *Past. tularensis* and *Trypanosoma lewisii*. The trypanosomal infection can of course be differentiated by microscopic study of the blood. In the case of the species of *Pasteurella* differentiation may be more difficult. *Past. muricida* produces indol and *Past.* hour broth cultures at 22° C., both in the case of the plague bacillus. *Past.* as do the other *Pasteurella*. For detailed discussion of the "hemorrhagic septicemia" diseases which do not affect man see "Manual of Veterinary Bacteriology" by Kelser and Schoening, Fourth Edition, 1943, Williams and Wilkins Company, Baltimore.

Pasteurella Tularensis

Habitat.—Primarily a disease of rodents but found to occur in a wide variety of wild and domestic animals and in man to whom it is transmitted by the bite of the deer-fly, *Chrysops discalis*, the tick, *Dermacentor andersoni* and other biting insects and by direct contact with infected animals as in the case of hunters and market people. The disease has been reported from all states of the Union and from the District of Columbia. It also occurs in many other parts of the world. Known by such names as "deer-fly fever" and "rabbit-fever."

Morphology.—Small, Gram-negative, non-motile, non-sporulating rods about 0.2 μ in thickness and from 0.3 to 0.7 μ in length, usually occurring singly. Pleo-clear area giving lesions or when clet.

Resistance.—Fairly susceptible to inimical agencies. Killed by moist heat at 55° to 60° C. in ten minutes.

Metabolism.—Optimum temperature 37° C. Optimum pH range 6.8 to 7.3. Grows well on coagulated egg and serum-dextrose-cystine agar; also on dextrose blood agar, dextrose serum agar, and blood agar slants, provided a piece of fresh, sterile rabbit's spleen is rubbed over the surface, and then left in the water of condensation.

Tularensis antiserum also agglutinates *Br. melitensis* and *Br. abortus* to about $\frac{1}{2}$ or $\frac{1}{4}$ of its titre; however, these organisms are unable to absorb agglutinins from a specific *tularensis* serum.

McAlpine and Brigham state that in 17 laboratories there have been 53 such accidents.

I. Examination of Clinical Materials.—McAlpine and Brigham state that three methods are available for the laboratory diagnosis of tularemia and list the agglutination test, culture and intracutaneous tests as the final tests to be used. They emphasize the extreme infectivity of virulent strains of *Past. tularensis* for laboratory workers, a characteristic also possessed by the genus *Brucella*, which incidentally has some serological relationship to *Past. tularensis*.

1. Microscopic.—Prepare films from the pathological specimen and examine for small Gram-negative, non-motile rods which may appear to be surrounded by a capsule and which are pleomorphic rods or cocci. Giemsa stain gives the best results with tissue preparations. While not too much can be hoped for from a microscopic examination of materials in this disease it is a good place in which to point out that microscopic examination of available materials is always indicated. It can be carried out while cultures are incubating or other tests are under way and it may produce positive or negative evidence of value if not finality. Thus, in the case of suspected tularemia, materials should also be stained by the Ziehl-Neelsen method to rule out the possibility of infection with *Myc. tuberculosis*, particularly in the case of an animal, naturally or experimentally infected.

2. Cultural.—Occasionally *Past. tularensis* may be isolated directly from a human case by culturing infected tissues or fluids on a suitable medium. It is considered better procedure, however, to inject the inoculum into guinea pigs, rabbits or white mice—preferably guinea pigs. Culture media may then be inoculated from the animals used after dispatching them with ether when death is impending. The animal thus serves in part as an "enrichment medium," although as will be seen its pathology serves to establish the diagnosis. Material for culture, from whatever source, is inoculated onto blood-dextrose-cystine agar and incubated at 37° C. After three to five days it is usually possible to find characteristic colonies in a positive case, but a tube or plate should not be considered negative until incubated for at least twenty-one days. Blood agar plates should also be inoculated with the original material in order to detect any other pathogenic organisms which may be present.

3. Animal Inoculation.—The following material may be used for the inoculation of animals:

- (a) Secretion from the sites of fly or tick bites.
- (b) Finger lesion exudate.
- (c) Conjunctival exudate.
- (d) Pleural effusion.
- (e) Peritoneal or spinal fluids.
- (f) Pneumonic sputum.
- (g) Suppurating glands of a patient within the first month of illness.
- (h) Spotted spleen or liver of infected animal.
- (i) Blood, drawn in the first week of illness, at least before the twelfth day (The blood is drawn, defibrinated and diluted 1 to 1 with saline.)
- (j) Parasites, such as ticks, suspected of carrying the infection.

The materials have of course to be properly prepared for injection into the guinea pig. This can be done by grinding in a mortar with saline and straining through gauze, following which a clear filtrate is made and the "filtrate" is made. In the case of the diluted, defibrinated blood the

injection is intraperitoneal and from 4 cc. to 8 cc. are injected. Where it is intracutaneous, 0.5 cc. of a 1% solution of the antigen in 0.5% saline solution is injected. In the case of a guinea pig, one guinea pig may be used for the purpose of obtaining material on the freshly

As a rule if *Past. tularensis* is present the inoculated animals will die in five to ten days. The characteristic lesions which should be looked for are hemorrhagic edema, but no pus at the site of inoculation; generally there are cervical, axillary or inguinal buboes, the glands being enlarged and filled with dry yellow caseous material; small white foci of necrosis of variable size in an enlarged dark spleen and liver; whereas the lungs are rarely involved. Smears made from these organs may not show *Past. tularensis* hence decision must be based on further studies of culture and

to the living organism and should not be placed in the same container with other organs.

4. Serology.—There are several immunological procedures to be considered in the diagnosis of tularemia.

(a) *The Agglutination Test.*—This test makes use of patient's serum and a known strain of *Past. tularensis*. Because of the high invasive power of this organism it is well to use a known avirulent strain such as Strain No. 38 distributed by the National Institute of Health. The test involves a series of serially diluted tubes of patient's serum to each of which a suspension of the known *Past. tularensis* is added. The tubes are agitated to effect mixture and incubated in the water bath at 45° to 55° C. for twelve to eighteen hours. Agglutination of *Past. tularensis* by serums in dilution of 1 to 80 or higher is considered diagnostic of tularemia, provided there is no cross agglutination with *Brucella*. Agglutinins appear in the patient's blood after the first week of the disease and usually increase rapidly and persist for a long time. If the serum under test is from a case of tularemia, the *tularensis* antigen will be agglutinated earlier and in more highly diluted serum than will the *abortus* antigen. If the serum is from a case of undulant fever the reverse may occur. The agglutination test may also be used to identify a pure culture suspected of being *Past. tularensis* by using suspensions of the culture under study and a known *tularensis* serum of determined titre. The workers should remember that freshly isolated *Pasteurella* species are dangerous to handle because of their very high invasive power.

(b) *The Bacterial Skin Test.*—In this test one injects into the skin of the flexor surface of the forearm enough of the antigen to make a skin wheal half a centimeter in diameter. The reaction does not usually appear until the thirty-sixth hour, hence readings are not made until forty-eight hours after injection. "A positive reaction resembles a positive tuberculin test—central, elevated, edematous indurations of about 1 cm. in diameter surrounded by an areola of erythema about 2 to 3.5 cm. in diameter. The size varies during the early stage of the disease, but after the eighth day the reactions are almost always of the size indicated." The antigen used is a polyvalent one prepared from at least six different strains by the special methods of Foshay who has pioneered in this aspect of serological

diagnosis. The test is still in the experimental stage but materials for it can be obtained from Dr. Foshay.

(c) *The Specific Antiserum Intradermal Test.*—This test is carried out with goat anti-tularensis serum diluted 1 to 10. Inject 0.1 cc. of this serum intradermally. According to Foshay the reaction, which is an erythematous-edematous response, usually appears promptly within two to four minutes, though sometimes delayed until ten to eleven minutes, and at the end of twenty minutes is 3 to 5 cm. in diameter and may be larger. It reaches its maximum in twenty-five minutes and then begins to fade. Like the bacterial intradermal test it is not always positive during the first few days of the disease. A control injection should be made using normal goat serum.

II. *Examination of Rodents and Insects.*—Rabbits or other rodents should be examined for the characteristic enlarged caseous glands, for white caseous nodules in the liver and spleen and for the other typical signs of *Past. tularensis* infection. The organisms may be identified by the procedures outlined for the examination of specimens from human cases. Specimens of flies, ticks or other insects suspected as transmitting agents may be examined by macerating them in a small mortar with saline and injecting the strained suspended material into a guinea pig.

III. *Differentiation of Tularemia from Plague.*—Tularemia is characterized by: (1) absence of pus at the site of inoculation in animals, (2) greater variability in size of the granules in the spleen, (3) rare lung involvement, (4) failure of the organisms to grow on ordinary culture media, and (5) specific agglutination reactions.

HEMOPHILUS

The genus *Hemophilus* is described thus: "Minute rod-shaped cells, sometimes thread forming and pleomorphic. Non-motile. Strict parasites growing best (or only) in the presence of hemoglobin and in general requiring blood serum, ascitic fluid, or certain growth accessory substances. Gram-negative."

Key to the Species of Genus *Hemophilus*

- | | |
|-------------------------------------|---|
| I. Affecting the respiratory tract. | 1. <i>Hemophilus influenzae</i> . |
| | 2. <i>Hemophilus suis</i> . |
| | 3. <i>Hemophilus hemolyticus</i> . |
| | 4. <i>Hemophilus parainfluenzae</i> . |
| | 5. <i>Hemophilus pertussis</i> . |
| II. Affecting the conjunctiva. | 6. <i>Hemophilus duplex</i> . |
| III. Affecting the genital region. | 7. <i>Hemophilus ducreyi</i> . |
| | 8. <i>Hemophilus haemoglobinophilus</i> . |

Where the relationship to growth accessory factors is known, the following summary may serve as a key:

Species*	Growth in peptone water containing		
	Hematin (%)	Yeast (%)	Hematin and yeast
<i>Hemophilus influenzae</i>	—	—	+
<i>Hemophilus suis</i>	—	—	+
<i>Hemophilus hemolyticus</i>	—	—	+
<i>Hemophilus parainfluenzae</i>	—	+	+
<i>Hemophilus haemoglobinophilus</i>	+	—	+

* The other species do not require the accessory factors for their growth.

Hemophilus Influenzæ

Habitat.—Found in the respiratory tract of man and once suspected as the cause of influenza.

Morphology.—Very 1
0.5 to 2 μ in length.
chains and in threads.
with dilute carbolfuchsin or Giemsa stain. According to some observers the bacillus
in its virulent smooth form is capsulated.

Blood.
37° C.

species and speak of the non-hemolytic and the hemolytic varieties of the influenza bacillus.

Chocolate Agar. Twenty-four Hours at 37° C.—Colonies similar to those on blood agar, but larger and much more profuse.

—Circular, translucent,
The surface is usually
the central granulation.

Blood Broth. Twenty-four Hours at 37° C.—Slightly turbid. No hemolysis.

Resistance.—Very sensitive to drying. Die rapidly in tap water. In dried sputum, vitality is retained from twelve to forty-eight hours. *Hem. influenza* is somewhat less resistant to heat than most other bacterial species, being killed in thirty minutes by exposure to a temperature of 50° to 55° C.

robe. Optimum temperature 37° C.

factor. Subcultures on plain agar, serum or ascites agar, gelatin, potato or milk fail to grow.

Biochemical Reactions.—*Hem. influenza* has no proteolytic action. Reactions on carbohydrates are variable, requiring specially prepared media. Most strains produce acid in levulose, galactose, dextrose, while only a small per cent ferment maltose, saccharose and dextrin. All strains negative on mannitol and lactose. Indol formed by some strains. Nitrates reduced to nitrites.

Serology.—*Hem. influenza* belongs to a heterogeneous antigenic group, making

There is no true toxin, but an endotoxin is produced.

Hemophilus Hemolyticus

As indicated above this non-pathogenic organism is very similar to the influenza bacillus and considered by some as merely a variety. It has much practical significance because it is very apt to be confused with *Strep. hemolyticus* in superficial studies of throat floras in which locale it is very much at home. Microscopic examination will of course reveal it to be a Gram-negative rod and not a Gram-positive coccus but on blood agar its colony and hemolysis simulate those of the *Streptococcus*.

Hemophilus Conjunctivitis

The organism, from the conjunctiva (pink eye) described by Koch and by Weeks and known as the Koch-Weeks bacillus was for a time described in the literature as *Hem. conjunctivitis*. It is now thought that it should be considered as a variety of *Hem. influenzae*. If the condition, "pink eye," is due to a variety of *Hem. influenzae* there must be factors of host resistance or pathogen specificity as yet unknown since this type of conjunctivitis is not very common whereas the influenza bacillus is frequently found in the nasopharyngeal region and at times is quite prevalent.

Hemophilus Duplex

Hemophilus duplex was earlier known as *Hem. lacunatus*. It is the Morax-Axenfeld bacillus found in sub-acute conjunctivitis. It requires neither the "V" nor the "X" factor and hence is not strictly speaking, a "hemophilic" organism.

Hemophilus Suis

This organism closely resembles the influenza bacillus except that it is relatively inert biochemically and differs immunologically. In association with a virus it is causally associated with swine influenza.

Hemophilus Haemoglobinophilus

This species is found in the preputial secretions of dogs. It closely resembles *Hem. influenzae* except that it requires only the "X" or hematin factor for growth.

Hemophilus Parainfluenzae

The parainfluenza bacillus requires only the "V" or yeast factor for growth and can therefore be cultivated on agar containing serum or ascites fluid. It is again another form closely resembling *Hem. influenzae*. It has been defined as non-hemolytic, but hemolytic strains showing the same nutritive requirements are found.

Hemophilus Ducreyi

Ducrey's "streptobacillus" is usually listed in the "hemophilic" group but this is largely convenience for though it is commonly grown in blood it does not specifically require the "V" or the "X" factors as such. The organisms, 1.5 μ in length by 0.5 μ in breadth, generally show a constriction at the middle. In the pus of ulcerating chancroidal lesions they occur singly, in pairs, or in small groups, while in culture they are often in chains and sometimes twisted together in dense masses. At the junction between the living and dead tissue of the lesion or in the medium, as when water of a culture medium, they appear as streptobacillary filaments and may occur as long chains. On blood agar in two to four days the colonies are small, round, slightly raised, grayish and opaque. After three or four days a whitish or light brown or yellowish point of dried serum is visible. Non-pathogenic to animals. It is the same as that which is the cause of chancroid.

Hemophilus Pertussis

This species, accepted as the etiological agent of whooping cough, is similar in morphology and staining properties to *Hem. influenzae* except that the bacilli are more uniform in size and pleomorphism is relatively uncommon. Unlike the "true hemophilic" bacteria, however, neither "V" nor "X" factors are required for its growth although when first isolated these factors favor its growth. At first similar on blood agar to the growth of the influenza bacillus its colonies later become larger, more opaque, and grayish or white in color and are surrounded by a narrow zone of hazy hemolysis.

On glycerol-potato-blood agar the colonies of *Hem. pertussis* are small, raised pearl-like colonies which are characteristic and larger and more opaque than those of the influenza bacillus. The organism is biochemically inactive. It does not form indol, does not reduce nitrates and fails to ferment any sugar. Unlike the influenza bacillus, *Hem. pertussis* is generally in the smooth state when isolated from the body on an optimal medium and it is immunologically homogeneous. It appears however that a collection of *pertussis* strains will reveal several (four, or more) groups, commonly called phases. These phases probably do not represent stable immunological entities like the types of pneumococci but rather successive stages in the "smooth-to-rough" transformation. Phase I (smooth, virulent) organisms are used in preparing effective vaccines. *Hem. pertussis* is consistently present in whooping cough. Its cell substance is toxic upon parenteral injection into experimental animals to about the same degree as is that of *Hem. influenzae*.

Examination of Clinical Materials.—1. Microscopic.—Make smears from freshly collected material and stain by Gram's method, also with dilute carbolfuchsin. Examine for characteristic morphology. Most important for the control of cultures obtained from colonies developing on appropriate media but also useful in examining pus, sputum, cerebrospinal fluid and soft chancre exudate.

2. Cultural.—The collected materials are planted on the media most suitable for the growth of the species suspected. After incubation for twenty-four, forty-eight and seventy-two hours, the plates are examined for growth characteristics, including the appearance of the colonies, morphology and staining characteristics of the bacilli. Final identification may include a determination of the accessory food factor requirement and consists in a study of the cultural reactions. It also takes into consideration the body site from which the infected material was obtained and the symptomatology, if any.

(a) *Hem. ducreyi* may be diagnosed by direct cultivation from a chancreoid lesion. The lesion is cleansed with sterile water or saline. Material is scraped from the bottom of the ulcer or from beneath its edges with a stiff platinum loop. This is planted in the medium (1 cc. rabbit blood per small tube, inactivated, after clotting, by heating to 55° C. for fifteen minutes) by passing the wire around the clot. After a day's incubation at body temperature the serum around the clot is stirred with a platinum loop and a spread made and examined by Gram's method. Characteristic chains of Gram-negative bacilli, sometimes in pure, sometimes in mixed culture will sufficiently identify the organism for practical purposes. An

unruptured bubo may be aspirated and material cultured as indicated above.

(b) *Hem. pertussis*.—The original medium of Bordet and Gengou is still used for the isolation of the organism causing whooping cough. The "cough plate method" is preferable to sputum culture and is carried out by holding an open Petri dish, containing the potato-glycerol-blood-agar medium, in front of the mouth during a cough paroxysm. The plates should be incubated in a humid atmosphere at 37° C. and when positive usually produce colonies in two days. Plates should, however, not be discarded as negative until incubated at least five days. Colonies of *Hem. pertussis* are larger, more opaque and whiter than those of the influenza bacillus. The Bordet-Gengou medium, as originally made, contained 50 per cent defibrinated blood. The use of so much blood is impossible in large scale work but is very desirable where it can be supplied. The amount of blood has been reduced to as small an amount as 16½ per cent (20 cc. blood to 100 cc. medium) by some workers who feel that with a proper salt balance less blood is needed. The Danish modification using 30 per cent blood has given excellent results but with it it is impossible to detect the hemolytic zone which is caused by the growth of *Hem. pertussis*.

(c) *Hem. influenzae*.—Many media have been devised for the cultivation of the influenza bacillus but the best appears to be heated blood

of a culture isolated from suspect material considers the source of the specimen, hemolytic properties and other cultural characteristics, including growth requirements. Thus the failure to grow on a suitable serum agar medium of a non-hemolytic strain from human source strongly suggests the influenza bacillus.

NOGUCHIA

A genus, named for Noguchi, the bacteriologist who isolated the type species, *N. granulosis*, of small, slender Gram-negative rods present in the conjunctiva of man and animals affected by a follicular type of disease, mucoid type of growth which on first isolation takes place with some difficulty in ordinary media; motile, flagellated, and encapsulated; aerobic and facultative anaerobic; optimum temperature for growth 28° to 30° C. Noguchi regarded *N. granulosis* as a cause of trachoma, but subsequent research tends toward a filterable virus etiology. Two other species, *N. simiae* and *N. cuniculi*, are recorded from *Macacus rhesus* monkeys and rabbits respectively. The genus *Noguchia* has little medical significance but illustrates well the fact that bacterial forms often occur in virus infections. There is a certain specificity in this phenomenon which poses some most interesting questions.

DIALISTER

In attempting to work out the etiology of a disease there is often an interesting panorama of scientific landmarks not usually recounted in the average textbook. This has been particularly true for tuberculosis, syphilis, and yellow fever, for instance. Influenza is now believed to be due to a

filterable virus. Until World War I, however, it was believed that *Hem. influenzae*, or Pfeiffer's bacillus, was the etiological agent of that disease. Evidence accumulated making such an assumption untenable and the search for the real agent began anew. In 1921, Olitsky and Gates described a tiny Gram-negative, non-motile obligate anaerobe which they found in nasopharyngeal washings from influenza patients in the early hours of the disease. The organism was grown under strictly anaerobic conditions in media containing fresh, sterile tissue or ascitic fluid. The organisms are minute, rod-shaped cells, occurring singly, in pairs, and short chains. These tiny organisms are in the group of "filter-passers." They are strict parasites but the early optimism that here lay the answer to the question of what causes influenza was not realized. The genus *Dialister* contains two species *D. pneumosintes* and *D. granuliformans* neither possessing more than passing interest.

ENTEROBACTERIACEÆ

The family *Enterobacteriaceæ* is composed of "Gram-negative rods widely distributed in nature. Many animal parasites and some plant parasites causing blights and soft rots. Grow well on artificial media. All species attack carbohydrates forming acid, or acid and visible gas (H_2 present). All produce nitrites from nitrates. When motile, the flagella are peritrichous." It is believed that the more logical treatment of the organisms of this family is to consider them together for they are closely related and it helps in considering the complex problem they present to realize this fact. There are 5 tribes containing 9 genera and 103 species. To this family of aerobic, non-spore-formers belong the organisms which cause typhoid and paratyphoid fever, bacillary dysentery, varieties of food poisoning, some summer diarrheas and urinary infections, and probably infectious diarrhea of the newborn. One genus, *Klebsiella*, contains species associated with pneumonia and other infections of the respiratory tract. The genus *Erwinia* is made chromogenic water bacteria

reptiles and fish. *Serratia* is the most famous of all chromogenic bacteria because of its historical connections with "miracles" and its widespread use in laboratories as an indicator organism. The genus *Proteus* contains urea-decomposing bacteria found in decaying material and occasionally in the bowel. The coliform bacteria, particularly the genus *Escherichia*, are considered the normal organisms of the adult intestine and are used in sanitary work as indicators of fecal pollution. The group as a whole has often been spoken of as the "colon-typhoid-dysentery" group. It is one of the most important and one of the most complex in medical bacteriology.

Key to the Tribes of Family Enterobacteriaceæ

- | | | |
|-----|---|-------------------------------|
| I. | tion of acid and visible gas. | Tribe I. <i>Escherichææ</i> . |
| II. | lactose with formation of acid, or acid and visible gas. Usually attack pectin. (One genus, <i>Erwinia</i> ; 13 species.) | Tribe II. <i>Erwinææ</i> . |

III. Chromogens producing a red pigment. Ferment dextrose and lactose with formation of acid, or acid and a small amount of visible gas. Liquefy gelatin. (One genus, *Serratia*; 6 species.) Tribe III. *Serratæ*.

IV. Ferment dextrose but not lactose with formation of acid and visible gas. Usually liquefy gelatin. Tribe IV. *Proteæ*.

V. Ferment dextrose with formation of acid, or acid and visible gas. Some ferment lactose with the formation of acid, but never visible gas. Usually do not liquefy gelatin. Tribe V. *Salmonellæ*.

ESCHERICHEÆ

This tribe comprises three genera. Two of these *Escherichia* and *Erobacter* are called "coliform bacteria." It is our belief that the third genus, *Klebsiella*, should also be so considered. It owes its high degree of individuality to the fact that it is recovered from pneumonia and other respiratory conditions. Actually it is difficult or impossible to distinguish from *Erobacter* or encapsulated *Escherichia* which occasionally occur. It would seem to us that *Klebsiella* merely represents coliform organisms which have exchanged their customary habitat in the bowel or even in the environment for residence in the respiratory tract which they maintain by virtue of their capsular defense. The wide variety of biochemical reactions of the *Klebsiella* favor this view although on the whole *Klebsiella* resemble *Erobacter* more closely and more commonly than they do *Escherichia*.

The recognized species of the tribe *Escherichæ* were formerly numerous, as late as 1934, 35 species being recognized by Bergey. At present 10 species are recog-

formerly

speciation

indol from tryptophane; the degree of acidity produced in glucose broth, the methyl red reaction; the formation of acetylmethylcarbinol (Voges-Proskauer reaction); and the ability to utilize citrate as a sole source of carbon. These four tests, in the order named, constitute the "IMVIC" reaction. In addition the liquefaction of gelatin, the production of hydrogen sulfide and the fermentation of glycerol and cellobiose are characteristics of use in classifying the members of the group. The fermentation of sucrose, salicin and dulcitol as well as the dissimilation of many other carbohydrates and carbohydrate-like substances no longer holds the significance it once did. Lactose is typically fermented with the production of acid and gas but strains are quite commonly encountered which produce acid and only a trace of gas, or only acid, or even which do not utilize lactose at all. Such cultures may of course be mistaken for members of the genus *Salmonella*. However, they may ferment saccharose and salicin and produce indol. Also the colony growth is more luxurious and serological tests will exclude them. It should be noted, however, that some coliform bacteria possess antigenic components similar to those found in the *Salmonella* but no coliform has yet been found which possesses both somatic and flagellar antigens similar to those found for *Salmonella*. These "atypical coliforms" or "paracolon" organisms which are aberrant with respect to lactose fermentation are a very real problem for they are frequently encountered in stool specimens and their precise significance is not established. Morgan's

bacillus, reported from summer diarrhea is at present classed in Bergey in the genus *Proteus*. This allocation does not have the unanimous support of all workers; we would include it as a coliform organism. The principal differential characteristics of the coliform bacteria, the tribe *Escherichæ*, are tabulated in Table 64.

TABLE 64.—DIFFERENTIATION OF COLIFORM BACTERIA

	Fermentation of		"In vivo reaction"				Liquefaction Gelatin	Production H ₂ S
	Dextrose	Lactose	Indol	Methyl red	Voges- Proskauer	Citrate		
<i>Escherichia coli</i>	AG	AG	+	+	—	—	—	—
<i>Escherichia freundii</i>	AG	AG	—	+	—	+	—	+
<i>Erubacter aerogenes</i>	AG	AG	—	—	+	+	—	—
<i>Erubacter cloacæ</i>	AG	AG	—	—	+	+	+	—
<i>Klebsiella</i> sp.	AG	AG	+	+	+	+	—	—

PROTEÆ

The tribe *Proteæ* has but one genus, *Proteus*, comprising 8 species. *Proteus morganii* occurs in normal stools, though but rarely, hence its presence in diarrheal stools may be significant. Five other species are listed in Bergey as "Habitat: Not known." Two species are said to occur in putrefying materials. One of these, *Proteus vulgaris*, has considerable medical significance. It has some pathogenicity in the urinary tract and it has been implicated in some cases of summer diarrhea, but it is chiefly significant because of its serological relationship to the etiological agent of typhus fever. Certain strains of *Proteus* (OX19, OX2, HXK, OXK) are made use of in the serum diagnosis of typhus and other diseases caused by *Rickettsia* in the Weil-Felix test. Also early work on "H" and "O" was done with *Proteus*. The genus *Proteus* are highly motile rods, hence normally when flagella are present they "swarm" over a plate having a moist surface. Non-motile strains occur and when plated these give discrete colonies which do not "swarm." Filamentous and curved rods are common as are also involution forms. Gram-negative. Aerobic. Proteins are decomposed. Ferment dextrose and sucrose with production of acid and gas but not lactose. This point differentiates *Proteus* from typical coliform bacteria but brings it into confusion, as far as lactose is concerned, with the *Salmonella*. Usually Voges-Proskauer negative. The production of ammonia from urea is an important characteristic, as is the formation of hydrogen sulfide. These two characteristics set off *Proteus* from all other Gram-negative gelatin-liquefying bacteria. It should be noted that *P. morganii* does not liquefy gelatin or ferment sucrose but it does produce hydrogen sulfide.

SALMONELLÆ

This tribe is made up of three very important genera, each of which includes species pathogenic for man, one includes animal pathogens, and in each there are species of no known pathogenicity.

Key to the Genera of the Tribe Salmonellæ

- I. Ferments dextrose with the formation of acid and usually gas.
Genus *Salmonella*.
- II. Ferments dextrose with the formation of acid but no gas.
 - A. Motile.
Genus *Elertella*.
 - B. Non-motile.
Genus *Shigella*.

Salmonella

The genus *Salmonella* is at the present time, with the possible exception of *Streptococcus*, our most complex genus of bacteria of medical significance. It contains four types of organisms: (a) those which are chiefly pathogenic for man, producing "para-typhoid" fever, such as *Sal. paratyphi* and *Sal. schottmuelleri*; (b) those which are pathogenic for animals and in man cause a variety of enteric fever or more commonly "food poisoning," such as *Sal. choleraesuis*, *Sal. enteritidis*, and *Sal. typhimurium*; (c) those pathogenic for animals only, as for example *Sal. pullorum*; and (d) a number of new types found in surveys, pathogenicity undetermined, such as *Sal. simsbury*. In this group the highest refinement of antigenic analysis in bacteriology has been achieved. At least 109 serological types are known, and this does not include coliform bacteria shown to contain *Salmonella* antigens. These types are identified through the use of somatic antigens of which there are 34, and flagellar antigens of which there are 64. From an immunological point of view the typhoid bacillus, *Eberthella typhosa*, is a *Salmonella*, but in practice bacteriologists continue to consider it as of a different genus (*Eberthella*).

Serologically *Salmonella* are placed in groups according to the immunological configuration of the soma or cell-bodies, i. e., according to the distribution of the "O" antigen. Thus Group A (containing only *Sal. paratyphi*) possesses "O" antigens II and XII. The mark of Group B is the possession of antigens IV and XII. At least 26 species (?) belong in group B including *Sal. schottmuelleri* and *Sal. typhimurium*. Group C is characterized by the possession of "O" antigens IV and VII and in Group C are placed 27 species (?) including *Sal. choleraesuis*. Seventeen species (?) up Group D thirteen species

III, X and XXVI in the cell body. At least seven other groups can be recognized but for practical purposes reasonable identification can be made of the most common *Salmonella* by biochemical studies combined with serology studies designed to place the organism in one of the first five groups. As far as we know the *Salmonella* types are relatively stable.

TABLE 65—BIOCHEMICAL AND AGGLUTINATIVE CHARACTERISTICS OF THE MORE IMPORTANT SALMONELLE¹

	Xylose	Arabi- nose	Lead acetate (H ₂ S)	Inositol	Treba- lose	Tar- trate	Dex- trose	Lac- tose	Antigenic group
<i>Sal. schottmuelleri</i> (Para. B)	AG	AG	+	AG	AG	Alk.	AG	—	B
<i>Sal. typhimurium</i> (<i>Sal. enteritidis</i>)	AG	AG	+	AG	AG	Acid	AG	—	B
<i>Sal. enteritidis</i>	AG	AG	+	—	AG	Acid	AG	—	D
<i>Sal. choleraesuis</i>	AG	—	—	—	—	Acid	AG	—	C
<i>Sal. abortusbovis</i>	AG	AG	—	—	AG	Acid	AG	—	B
<i>Sal. hirschfeldii</i> (Para. C)	AG	AG	+	—	V		AG	—	C
<i>Sal. paratyphi</i> (Para. A)	—	AG	—	—	AG	Alk.	AG	—	A

¹ BERGEY: Manual of Determinative Bacteriology, 5th ed., Baltimore, Williams & Wilkins Company, 1939.

It is obvious that such work as complete *Salmonella* typing cannot be carried out by the average laboratory. *Salmonella* Centers have been set

up in various parts of the world to meet this problem. P. R. Edwards has long been a leader in this field in America and under him at the University of Kentucky has been developed the largest of the American centers. Outstanding work abroad involves the names of Kauffman and Bruce White. Cultures which require special facilities for typing may be sent to the centers maintained by the Army or to Dr. Edwards. In a recent survey Edwards and associates found that 3090 cultures of *Salmonella* from 2285 outbreaks of infection in man and animals fell into 59 groups or types. The problem is not hopelessly complex, however, for it is possible to identify with reasonable accuracy most of the *Salmonella* cultures encountered. In Table 65 are listed the essential biochemical and serological characteristics of the more important *Salmonella* species. The type species of the genus is *Sal. choleraesuis*.

Salmonella Choleraesuis

Habitat.—Found frequently in swine in which it occurs both in the presence and
Occa-
type

at

no.

Agar Plate. Twenty-four Hours at 37° C.—Colonies round, moist, grayish in color, translucent, with smooth surface.

Eosin-Methylene Blue Agar Plate. Twenty-four Hours at 37° C.—Colonies are round, moist, translucent, colorless or pinkish in appearance. May later have a bluish tint.

rowth, grayish in color.

ndity with thin surface

. for thirty minutes.

rough and mucoid variants may be produced.

Salmonella Typhimurium

This species, often called *Sal. artrycke*, is in the United States, the most widely distributed of all the *Salmonella* types and is probably able to attack all species of warm blooded animals. With the exception of *Sal. schottmuelleri* it occurs more frequently in man than any other type. *Sal. typhimurium* belongs to the antigenic group B and has the formula IV,

XII:i:1,2,3. As an animal strain it produces acid in tartrate, it forms hydrogen sulfide, and it ferments xylose, trehalose, arabinose and dulcitol. *Sal. paratyphi* does not ferment xylose, produce hydrogen sulfide or acidify tartrate agar. Although *Sal. choleraesuis* ferments xylose it fails to ferment trehalose, arabinose and dulcitol and in one phase it does not produce hydrogen sulfide. *Sal. choleraesuis* acidifies tartrate medium. *Sal. enteritidis* is biochemically similar to *Sal. typhimurium*. *Sal. schottmuelleri* does not acidify tartrate but unlike *Sal. paratyphi* it ferments xylose and produces hydrogen sulfide.

Salmonella Paratyphi

This species of *Salmonella* has the antigenic formula Group A:I, II, XII:a:—. As indicated it exists in only one phase with respect to the "H" antigen. *Sal. paratyphi* causes paratyphoid fever and with rare exceptions, possibly questionable, is found only in man. In the paragraph above it is indicated that this species fails to acidify tartrate medium, produce hydrogen sulfide or ferment xylose. These reactions will identify the organism for practical purposes. Incidentally it is the only species in serological group A. It is now less commonly encountered in the United States than formerly.

Salmonella Schottmuelleri

This species is found in animals and in man. The strains found in animals acidify tartrate and are spoken of as the "animal type," those found in man fail to change the reaction in tartrate medium but unlike *Sal. paratyphi* which is also tartrate negative *Sal. schottmuelleri* ferments xylose and produces hydrogen sulfide. It produces paratyphoid fever in man but is sometimes recovered from gastro-enteritis. Its serological formula is Group B:I, IV, V, XII:b:1, 2 although somatic components 1 and V are sometimes missing.

Salmonella Enteritidis

The serological formula of this organism is Group D:I, IX, XII:g.m.:— The typhoid bacillus, when considered as a *Salmonella* species is Group D:IX, XII (Vi):d.:—. Thus it will be seen that a close serological relationship exists between the typhoid bacillus and such organisms of the genus *Salmonella* as *Sal. enteritidis*. At the present time 15 species other than *Sal. enteritidis* belong to this Group D and may be confused with the typhoid bacillus. Included here are *Sal. panama*, *Sal. dublin*, and the organisms causing fowl typhoid and diarrhea of chicks, diseases not transmissible to man. The need is thus shown for care in the interpretation of serological findings. *Sal. enteritidis*, as mentioned above, is culturally like *Sal. typhimurium*. Its immunological difference is clear since one belongs to Group B, the other to Group D. *Sal. enteritidis* occurs very rarely in animals other than rodents. This indicates that food infection by this species probably has its origin in rodents.

Salmonella Hirschfeldii

In Group C, formula: VI,VII,(Vi):c:1,5 occurs "paratyphoid C." In common parlance *Sal. paratyphi* is "para A" and *Sal. schottmuelleri* is "para B." The "para C" was not encountered once in the extensive

Edwards survey. It has been reported as common in countries bordering the Carribean and in parts of Europe. Culturally *Sal. hirschfeldii* has not received the attention accorded more important members of the genus. It gives the same fundamental "reactions" as *Sal. typhimurium* except that it does not ferment inositol which may be fermented by *sertrycke* and since the Hirschfeld strain appears to be a human strain it will not change the reaction of Jordan's tartrate medium.

EBERTHELLA

The genus *Eberthella* is described thus: "Gram-negative rods. Attack a number of carbohydrates with the formation of acid but no gas. Do not form acetylmethylcarbinol." There are 14 listed species, all motile, of which only one, *Eberthella typhosa*, the cause of typhoid fever, need concern us. With the exception of one species, *Eb. xenopa*, which is associated

The recognition of a culture as *Eberthella typhosa* is therefore not complete until the organism has been thoroughly scrutinized, including serological confirmation. This is particularly true because of the fact that the continued fevers, to which typhoid belongs, are a complex and difficult group. It would be entirely possible to isolate from the bowel contents of such a case an organism which might be called the typhoid bacillus after only superficial tests, when the patient actually might be suffering from typhus, brucellosis, tuberculosis, endocarditis or other disease of the continued fever group.

Eberthella Typhosa

Habitat.—The intestinal canal and blood stream of patients ill with typhoid fever. The urine, bile and feces of carriers.

Granular, brown, opaque, ...
granularities of surface

—Freshly isolated strains

... slightly spreading, barely visible growth

Gelatin. Twenty-four Hours at 20° C.—Grayish, transparent to opaque.

Gelatin Stab Twenty-four Hours at 20° C.—Thin, white, opalescent surface growth. No liquefaction.

Shigella Dysenteriae

Habitat.—Intestinal canal of bacillary dysentery patients or of carriers.

Morphology.—Rods or cocco-bacilli 0.4 to 0.6 μ by 1 to 3 μ , occurring singly. Non-motile, non-flagellated, non-capsulated and non-sporulating. Gram-negative and non-acid-fast.

Agar Plate. *Twenty-four Hours at 37° C.*—Colonies round, grayish, with edges

ty-four Hours at 37° C.—Colonies, small, appearance. If the dye is too concen-

Gelatin Stab. *Twenty-four Hours at 20° C.*—Grayish surface growth. No liquefaction.

Broth. *Twenty-four Hours at 37° C.*—Slightly turbid with grayish sediment.

Resistance.—Killed by moist heat at 60° C. in thirty minutes. Usually dies in

Metabolism.—Aerobe and facultative anaerobe. Optimum temperature, 37° C.

and then slightly alkaline. Indol —; nitrates not reduced; H₂S —; citrate —; V. P. —; NH₃ +; methylene blue reductase +. It produces a powerful soluble toxin.

Serology.—*Sh. dysenteriae* (Shiga) is antigenically homogeneous and distinct.

Pathogenicity.—Causes bacillary dysentery in man. The soluble toxin when lapse and paralysis of

the smooth type, but later rough variants may be produced.

Shigella Paradysenteriae

Shigella paradysenteriae (the Flexner "group") is a species which is characterized by its cultural and serological heterogeneity in contrast to *Sh. dysenteriae* which is homogeneous. This has resulted in a number of efforts to define the various types which compose the species of which the best known are the Flexner, the Strong and the Park-Hiss-Russell. Subdivisions made upon a fermentative basis were found to be inconstant and are now only of historical interest. For more than a decade a satisfactory working basis for determination of five types V, W, X, Z and Y, has been that of Andrewes based upon the relative distribution within the cell body of the four antigenic factors they contain, namely V, W, X and Z. The type is named from the antigen present in preponderance, type Y being that form in which all four components are present in approximately equal amount. Recent studies by Boyd, also on an immunological basis, present a new classification of type already adopted by the British Army. Boyd does not believe that types Y and X are independent types. He retains the Andrewes V, W and Z and adds three new types. These then are called Flexner I, II, III, IV, V and VI. The new I, II and III correspond respectively to V, W and Z. Boyd studied 7339 strains of dysentery bacilli in India, 1932–1935, and found that 67.5 per cent were of this Flexner species. Comparable data for other countries do not exist but it is known that in the United States a similar importance of *Sh. paradysenteriae* obtains which is shared with *Sh. sonnei*, a milder but possibly more common excitant of dysentery. Recent epidemics caused by Shiga's bacillus have also been reported.

Shigella Ambigua

This species, the "Schmitz" bacillus, resembles *Sh. dysenteriae* in all important cultural characteristics except indol formation which is positive for *Sh. ambigua* and negative for *Sh. dysenteriae*. They are immunologically distinct. This organism is not an important cause of dysentery in the United States but it plays a more important rôle in the Far East.

The Newcastle Type

This organism was isolated from a case of diarrhea in Newcastle-upon-Tyne in 1925 and possesses two peculiarities: (1) occasionally a slight bubble of gas is produced from dextrose and dulcitol; and (2) when the substrate is dissolved in beef extract broth, dextrose, dulcitol and maltose are always fermented to acid and gas. Although rated as a species by Bergey it has never been given a specific epithet. Weil states that the Newcastle type has been found in dysentery cases sporadically or in epidemics "over the whole earth." Boyd feels that the Newcastle bacillus is really a Flexner type and he places it along with some other strains encountered in his work in India in the classification of Flexner types mentioned above as "Flexner VI."

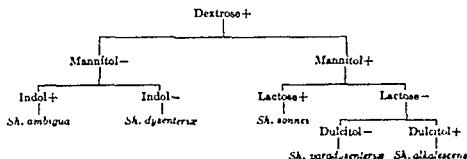
Shigella Sonnei

Several species of *Shigella* slowly form acid from lactose. Of the five which have this property only *Sh. sonnei* has much medical significance. The bacteria formerly called *Sh. dispar* have been assigned to *Sh. ceylonensis*, *Sh. madampensis* and *Sh. sonnei*. Sonne's bacillus does not form indol which differentiates it from the other two former species. It is well known that "Sonne dysentery" is one of the most common forms of bacillary dysentery at the present time. It is not true that it is always mild for some of the dysentery in Japan of greatest severity is of *Sh. sonnei* etiology.

Shigella Alkalescens

This species was described in 1918 for the sake of its differentiation from *Sh. paradysenteriae*. It does not fail to produce acid from certain sugars; its name comes from its end action on milk. Neter has shown that *Sh. paradysenteriae* is a more common cause of infections of the bowel as a complication of the history of diarrhoeic diseases. The more useful reactions of the *Shigella* group are summarized in Table 66.

TABLE 66—CHARACTERISTICS OF IMPORTANT SPECIES OF SHIGELLA



Examination of Clinical and Other Materials for Bacteria of the Family Enterobacteriaceæ.—Typhoid and paratyphoid organisms may be isolated from the blood, feces, urine or bile of cases of acute enteric fever caused by these organisms. In carriers the organisms may be found in the bile, feces or urine. In the enteric fevers it is occasionally possible to isolate the organisms from other materials including the skin, pus, spinal fluid and sputum. At times they may be obtained from contaminated water, milk, shellfish or foods. The organisms causing food poisoning and food infection and their isolation will be considered in a separate section. (See Chapter XXVII.) In bacillary dysentery, the organisms are usually present only in the feces, and therefore only fecal materials are used for examination. Coliform organisms, members of the genus *Proteus* and *Shigella alkalescens* may be found in urine. *Klebsiella* may be recovered from pus, sputum and blood.

1. **Microscopic.**—Microscopic examinations have practically no diagnostic value, except for the determination of motility, since all of the organisms are Gram-negative, non-spore-bearing short rods. The question of motility may be of importance in distinguishing the motile typhoid organisms from the non-motile dysentery bacilli, but sometimes non-motile typhoid strains occur. *Klebsiella* is usually encapsulated but the colony type and mucoid, stringy growth suggest the presence of capsules and it should be recalled that capsules are not always demonstrable, particularly in organisms long under cultivation.

2. **Cultural.**—It would seem to be a simple matter to cultivate members of the family *Enterobacteriaceæ*, since practically all of them grow readily. To obtain a pure culture for detailed study is quite another proposition particularly from feces, because the pathogen sought in the bowel is mixed with a great number of other bacteria. The problem then is to prepare a medium which will inhibit the dissimilar admixed bacteria and indicate in some way the organisms sought for. The simplest mediums divide the coliform bacteria from the *Salmonella-Proteus-Eberthella-Shigella* organisms through the use of indicator lactose-agars. Lactose-litmus, Endo's and eosin-methylene blue agars are illustrations of this technic. The addition of other chemicals to specifically encourage the organism sought at the expense of those not sought and to help identify it in some way represents a further advance. A large number of such culture media have been prepared. Good results depend upon expert use of properly prepared media—there is no short cut. Among these special media are the Wilson-Blair bismuth-sulfite agar, the *Shigella-Salmonella* thiosulfate-citrate-bile agar, desoxycholate citrate agar, and MacConkey's bile salts agar. The enteric worker should try these media and choose the ones he works best with. Possibly one might recommend eosin-methylene blue for routine plating and "S-S." agar for use in plating for recovery from specimens submitted for examination. Given a good fresh specimen the experienced worker will have no trouble in obtaining the pathogen sought on so simple a medium as lactose litmus agar. However such optimal conditions do not often exist and it is best to use the most efficient medium obtainable. Specimens of feces or urine may be added to 30 per cent glycerol-saline or to a solution of selenite "F" for transport to a laboratory for examination. Sometimes a specimen, negative on direct plating, gives positive results after being held in such an enrichment medium as selenite "F." Blood cultures may

be made using bile broth, Kracke's medium, nutrient broth or brilliant green broth. Bile media are thought to encourage the growth of the typhoid bacillus but if used the blood should also be cultured in non-bile media since in advance of results one cannot be sure the case is one of typhoid.

TABLE 67.—REACTIONS OF GRAM-NEGATIVE INTESTINAL BACILLI ON EOSIN-METHYLENE BLUE AGAR, RUSSELL'S DOUBLE SUGAR MEDIUM, SIMMONS' CITRATE AGAR AND JORDAN'S TARTRATE AGAR

Organisms	Eosin-methylene blue agar	Russell's double sugar		Simmons' citrate agar	Jordan's tartrate agar
		Bott	Slant		
<i>Escherichia coli</i>	Large discrete colonies with large, dark almost black centers, with greenish metallic sheen	Acid Gas +++	Acid	No growth or colorless pinpoint colonies, medium unchanged	
<i>Aerobacter aerogenes</i>	Large mucoid colonies with small, dark brown or black centers, rarely show metallic sheen	Acid Gas +++	Acid	Heavy opaque milky growth, medium blue	
<i>Salmonella paratyphi</i>	Translucent, colorless or pinkish colonies, similar to typhoid, later have bluish tint	Acid Gas +	Alkaline	No growth, medium unchanged	Medium alk. (red)
<i>Salmonella schottmuelleri</i>	Translucent, colorless or pinkish colonies similar to typhoid, later have bluish tint	Acid Gas +	Alkaline	Heavy, translucent growth, medium blue	Medium alk. (red)
<i>Salmonella enteritidis</i>	Translucent, colorless or pinkish colonies similar to typhoid, later have bluish tint	Acid Gas +	Alkaline	Heavy, translucent growth, medium blue	Surface alk. (red), butt acid (yellow)
<i>Salmonella antrax</i>	Translucent colorless or pinkish colonies similar to typhoid, later have bluish tint	Acid Gas +	Alkaline	Heavy, translucent growth, medium blue	Surface alk (red), butt acid (yellow)
<i>Enterella typhosa</i>	Translucent, colorless or pinkish colonies	Acid	Alkaline	No growth, medium unchanged	Surface alk (red), butt acid (yellow)
<i>Shigella dysenteriae</i>	Small translucent, colorless or pinkish colonies	Acid	Alkaline	No growth, medium unchanged	
<i>Shigella paradyenteriae</i>	Small translucent, colorless or pinkish colonies	Acid	Alkaline	No growth, medium unchanged	

Should growth appear in the broth to which blood has been added subculture should be made to Endo's or eosin-methylene blue agar to obtain pure cultures for final identification. Colonies appearing on plates made from feces should be suspended in saline or broth and replated to ensure a pure culture because on such inhibitory mediums as Wilson-Blair heavy

inoculations are made and it is entirely possible that the small glistening black colony on a brown background picked off a Wilson-Blair plate as a probable *Eberthella typhosa* may have mixed with it other bacteria from the original specimen which did not grow but which may develop in a more favorable medium, giving rise to a mixed culture. When convinced that a pure culture is in hand subcultures are made, usually to Russell's double sugar and plain agar slants. Within twenty-four hours the Russell's slant will indicate the group in which the organism is to be found: (1) coliform, (2) *Salmonella* or *Proteus*, (3) *Eberthella* or *Shigella*, or (4) Fermentation reactions negative, as encountered for example with *Alcaligenes faecalis*, a species of the family *Rhizobiaceæ*, whose habitat is said to be the intestinal canal. From the agar slant subcultures may be made as indicated to effect final cultural identification and from it also suspensions may be prepared for the serological work deemed necessary. (See Table 67.)

3. Serological.—(a) *Culture Identification*.—The results of the biochemical reactions should be confirmed by agglutination and, if necessary, by agglutinin absorption tests. The macroscopic tube-agglutination test is usually used.

Prepare a uniform suspension of the unknown organism by mixing the entire twenty-four hour growth on an agar slant with about 6 cc. of saline. Remove all clumps by slow centrifugation for a few seconds or by pushing a pledget of cotton slowly to the bottom of the tube allowing the homogeneous suspension to "filter up" through the cotton. Dilute the suspension to the opacity of tube No. 3 of the McFarland nephelometer, and use 0.5 cc. amounts of this suspension in the agglutination tests. Set up parallel tests with 0.5 cc. amounts of the various dilutions of specific antisera indicated, using for each antiserum sufficient dilutions to show the end titres of the respective sera.

(b) *Widal Test*.—One of the most valuable and widely used diagnostic serological tests is the Widal test in which the blood serum of a patient suspected of typhoid factors.

paratyphoid and this results in antibody formation. Again, as we have noted in the section on *Salmonella*, one of the serological groups of that genus has the same somatic antigen configuration as the typhoid bacillus. Persons who have recently had an infection with a paratyphoid of Group D may therefore have "O" antibodies which will react with the typhoid bacillus. At the present time these difficulties are surmounted by the "Qualitative Receptor Analysis" method in which the patient's blood serum is tested against typhoid antigen "O" and typhoid antigen "H." Definite infection may be indicated by a titre of 1 to 160 or higher with "O" antigen and 1 to 80 or higher with "H" antigen. An anamnestic reaction, past infection, or recent immunization may be indicated by a titre of 1 to 160 or under with "H" antigen only. Negative results may be due to sampling the patient's blood before the appearance of agglutinins in the serum so that the later appearance of agglutinins after a negative result is usually significant. If only "O" agglutination is obtained ("H" being negative) the serum should be checked against *Sal. enteritidis*. If it is agglutinated the infection is due to *Sal. enteritidis* and not to the typhoid

bacillus. The diagnostic method of choice in suspected typhoid fever before the agglutinins appear is of course the blood culture. It should be possible to make a diagnosis of typhoid either by blood culture or Widal test before the organisms can be readily recovered from the urine or the feces. Examining these excretions should be largely by way of further confirmation or for purposes of determining the end of the period of infectivity or for establishing the carrier condition. It is interesting to note that typhoid carriers usually show the "Vi" antibody in their serum which can be tested for with a "Vi" antigen.

The Widal type of reaction is not useful in bacillary dysentery or food infection since these diseases do not take the form of a "continued fever" and the blood serum content of antibody is irregular and low.

4. **Animal Inoculation.**—Animal inoculation is not utilized in routine diagnostic work with bacteria of the family *Enterobacteriaceae*.

BRUCELLA

The genus *Brucella*, as at present constituted, consists of four species which are described as "Minute rods with many coccoid cells, $0.5\ \mu$ by 0.5 to $2.0\ \mu$; motile or non-motile; Gram-negative; gelatin not liquefied, neither acid nor gas from carbohydrates; parasitic, invading animal tissue, producing infection of the genital tract, the mammary gland or the lymphatic tissues, the respiratory and intestinal tracts; pathogenic for various species of domestic animals and man."

The described species include *Brucella melitensis* (type species), *Br. abortus* and *Br. suis* the causative agents of the caprine, bovine and porcine varieties of undulant fever in man and *Br. bronchiseptica*, claimed by some to be the cause of distemper in dogs and the cause of acute infection in cats, rabbits, guinea pigs, ferrets, white rats and monkeys.

Key to the Species of Genus *Brucella*

1. Non-motile:

A. Grow in media containing basic fuchsin.

(1) Grows in media containing thionin.

Brucella melitensis.

(2) Does not grow in media containing thionin.

Brucella abortus.

B. Does not grow in media containing basic fuchsin.

(1) Grows in media containing thionin.

Brucella suis.

Brucella bronchiseptica.

2. Motile.

Brucella Melitensis

Habitat.—Primarily a strict parasite of goats, but may be transmitted to man.

—Colonies small, round, about $0.5\ \text{mm}$.
ng surface and entire edge. By reflected

light, translucent, grayish-white in color, while by transmitted light they are almost colorless. Consistency butyrous and easily emulsified. Older colonies are slightly larger and grayish-yellow in color

grayish-white
n of the tube.

turbidity, no
growth with

moderate turbidity, and a moderate deposit which becomes viscous and almost impossible to disintegrate.

color, not as a rule by blood and serum. Brown pigment formed on potato and old agar cultures. Growth in all media is relatively slow. Does not produce hemolysin.

Biochemical.—No carbohydrates fermented, but 5 to 20 per cent of glucose is

lutions will cross agglutinate
glutinate *P. tularensis*.

chronic septicemic infection
fected by feeding or by sub-
oratory infections of humans,
uncommon.

Brucella Abortus

This organism, which was isolated by Bang, in 1879, from cows with infectious abortion and called *Bacillus abortus*, is now recognized as a member of the genus *Brucella*. It causes contagious abortion in cattle and other animals and has been established as one of the causes of undulant

and grows better in the presence of 5 to 10 per cent CO₂ although old cultures do well in the air. For its isolation the culture tubes or plates may be placed in a glass jar filled with 10 per cent CO₂. A simple method for inoculation, is by igniting the cotton tube with a cork soaked in paraffin.

2 per cent of glucose, and when grown on media containing sulfur it gives off detectable amounts of H₂S. It produces specific agglutinins and complement-fixing bodies but cross reactions with the related species are apt to cause some confusion.

For the differentiation of the three species Huddleson recommends the use of 2 media made by adding to beef liver infusion agar (pH 6.6) one of the following dyes: (1) thionin, 1 to 50,000; (2) basic fuchsin, 1 to 25,000; pyronin, 1 to 100,000 may be used in addition. The growth of *Br. abortus* is inhibited by the thionin medium but not by the others. The differential characteristics of species of genus *Brucella* are shown in Table 68.

Br. abortus, when injected subcutaneously or intraperitoneally into guinea pigs, produces a chronic, non-fatal infection, with focal lesions in

the internal organs, especially in the glands, spleen, liver and testicles. Most of the brucellosis in this country is of the bovine type.

Br. abortus infection is said to be less virulent for humans than that caused by *Br. melitensis* or *Br. suis*.

TABLE 68.—DIFFERENTIAL CHARACTERS OF THE THREE RELATED SPECIES OF GENUS BRUCELLA

Species	10 per cent CO ₂ required for primary isolation	H ₂ S forma- tion (days)	Growth on media containing	
			Thionin	Basic fuchsin
<i>Br. melitensis</i>	0	≈ 1	+++	+++
<i>Br. abortus</i>	++	2	0	+++
<i>Br. suis</i>	0	4	+++	0

Brucella Suis

This organism, which infects swine, other animals and man, resemble the other two species quite closely. Methods similar to those mentioned above are used for its identification. It grows well on Huddleson's thionin medium but is inhibited by the basic fuchsin and by the pyronin.

I. **Examination of Clinical Materials.**—The laboratory diagnosis of undulant fever may be established by isolating the causative organism from the blood, milk (animals), urine or feces either by culture methods or by animal inoculation; by the demonstration of specific antibodies and active specific opsonin in the serum; and by skin tests.

1. **Microscopic.**—The morphological characteristics of the organisms are not sufficiently distinctive to enable one to identify them in smears prepared from pathological lesions. However, the finding of small Gram-negative rods with many coccoid forms may be of assistance as a step in the isolation of the organism.

2. **Cultural.**—While the organisms may be found in the blood during the early stage and during febrile periods of the disease, the results of cultures are often disappointing. Collect 10 cc. or more of blood and inoculate this into flasks containing 250 to 500 cc. of nutrient infusion broth or liver infusion broth. Incubate at 37° C. and observe for at least three weeks, making frequent transfers to duplicate sets of plates containing glycerol agar, chocolate blood agar and liver infusion agar. Incubate one set of the plates in a jar filled with 10 per cent CO₂. Examine the plates daily for typical slow-growing small transparent colonies and transfer these to fresh media to secure a pure culture. Identify the species by determining its various cultural, biochemical and serological reactions.

Urine or milk specimens may contain the organisms at irregular intervals during the disease. In making cultures of urine, centrifugalize enough of a fresh catheterized specimen to obtain several loopfuls of sediment. Inoculate this in the media and incubate as directed above. A similar procedure may be used in examining milk but both the sediment and the fat should be used to inoculate the media. The pure cultures may be tested to determine their ability to grow on Huddleson's differential dye media.

3. **Serological.**—(a) *Confirmation.*—A pure culture of the suspected organism may be identified by macroscopic tube agglutination tests using anti-sera prepared with *Br. melitensis*, *Br. abortus* and *Br. suis*, respectively. It may be necessary to resort to the agglutinin absorption test to determine the species.

(b) *Agglutination Test*.—Because of the irregular results obtained in attempting to isolate the organism from the blood or urine, the clinical diagnosis of undulant fever is commonly buttressed by several immunological procedures. One of these is the demonstration of specific agglutinins in the patient's serum. Using the macroscopic tube-agglutination method test the serum against antigens prepared with pure cultures of *Br. melitensis*, *Br. abortus*, *Br. suis* and also with *Past. tularensis*. There is some doubt

exceeds 1 to 80. If different antigens are agglutinated by the patient's serum in approximately the same dilutions it may be necessary to carry out agglutinin absorption or complement-fixation tests. Consequently many clinical laboratories limit the examination to the recognition of agglutinins for organisms of one species of the *Brucella* group, usually *Br. abortus*.

(c) *Opsono-Cytophagic Test*.—The capacity of the neutrophile polymorphonuclear leucocytes of the patient's blood to ingest organisms of the genus *Brucella*, presumably an expression of the specific opsonin content of the serum, is measured and the data utilized in determining the presence or absence of infection and of the immune state. The test consists in mixing small equal amounts of the patient's citrated blood and of a freshly prepared suspension of a known smooth strain of *Brucella*. The mixture is incubated in the water bath for half an hour at body temperature. Then a drop of the sedimented cells is withdrawn by means of a capillary pipet and placed on a slide. The drop is spread as in the preparation of a blood smear and stained after fixation. A routine blood stain such as Wright's may be used. Hitchens and Sullivan advise, however, that the slide be immersed for fixation three minutes in a solution containing 1 per cent acetic acid and 5 per cent formalin in distilled water. They then would stain for fifteen seconds with carbol toluidin blue. The slide is then examined microscopically and 25 isolated polymorphonuclear leucocytes from different parts of the film are studied. In case the leucocytes contain no more bacteria than are distributed in corresponding areas outside the cells the test is recorded as negative. "Slight" phagocytosis is the reading for the leucocytes which contain more bacteria than the surrounding fields but are not "marked" which is the expression for the leucocytes which are "filled" (containing 40 or more bacteria). Interpretation of these results, as well as those from the agglutination and skin tests is indicated in Table 69.

TABLE 69.—A PROPOSED SYSTEM FOR THE DIAGNOSIS OF UNDULANT FEVER ACCORDING TO THE RESULTS OF THE AGGLUTINATION, ALLERGIC AND OPSONO-CYTOPHAGIC TESTS
(Huddleson, Johnson and Hamann, Am Jour. Pub. Health)

Agglutination test	Allergic skin test	Opsonophagic power of blood	Status toward <i>Brucella</i>
—	—	Cells negative to 20 per cent slight	Susceptible
—	+	Cells negative to 40 per cent marked	Infected
—	+	Cells 60 to 100 per cent marked	Immune
+	+	Cells 60 to 100 per cent marked	Immune
+	+	Cells negative to 40 per cent marked	Infected

(d) *Skin-test*.—Brucellergin is prepared from smooth strains of *Br. abortus*, or other species of the genus after they have been extracted with ether. The extracted, dry cells are ground to a powder and "dissolved" in distilled water. From the solution the protein nucleate is precipitated by acetic acid adjustment of the pH to 4.5. The precipitate is obtained and purified by a series of dissolving and reprecipitating procedures and is finally made up in 1 per cent solution at pH 6.8 filtered and preserved with phenol (final concentration 0.5 per cent). This is kept in sterile bottles in the cold room and for use in humans has to be standardized in rabbits. The stock solution which was obtained by NaOH solution of the protein nucleate is treated with HCl until cloudy. Dilutions are made in saline of the suspended material and 0.1 cc. of each dilution is injected intradermally into sensitized rabbits (made sensitive by intravenous injection of 1 cc. of 1 to 100 dilution of a virulent culture of *Brucella* from a forty-eight hour agar slant culture; skin sensitiveness develops in the rabbit in about thirty days). The rabbit reactions are read after forty-eight hours. The dose to be used in humans is the lowest dilution of the nucleoprotein suspension (brucellergin) which produces a skin reaction 5 mm. in diameter in sensitized rabbits.

The test in the human is carried out using 0.1 cc. of the proper dilution of the brucellergin, as determined in the rabbit. This amount is injected intradermally and the reaction is read at twenty-four and at forty-eight hours. Persons who have not been sensitized to *Brucella*, and who are probably susceptible to infection, show no local or systemic reaction although in normal individuals an erythema without edema may appear about the point of the injection. Such non-specific reactions subside between the twenty-four and forty-eight hour readings, while at this period specific reactions if present would increase. A true reaction is marked by erythema and slight edema involving an area with a diameter from 2 to 10 cm. It may persist for forty-eight to ninety-six hours and occasionally even longer. In infected individuals the local reactions may be accompanied by more marked manifestations of symptoms; focal reactions especially may be noted. Hypersensitive persons may respond with severe systemic reaction. (See Table 69.)

4. *Animal Inoculation*.—The organism may be isolated from infected tissues, blood, urine or milk by inoculating such materials subcutaneously or intraperitoneally into each of two or more guinea pigs. If the animals do not die, kill them at the end of seven weeks and examine the internal organs, especially the lymph glands, liver and spleen, for small grayish lesions which may resemble those of tuberculosis. From such lesions prepare smears and stain them by the Gram and Ziehl-Neelsen methods. Also make cultures by rubbing the infected tissues over the surface of solid media; incubate one set of the plates aerobically and the other in an atmosphere containing 10 per cent CO₂. Isolate and identify the organisms as outlined above.

II. *Examination of Materials from Animals*.—*Br. melitensis* infection in goats may be recognized by cultivation of the organism from the milk, blood or urine but as a rule the diagnosis is made on the basis of the demonstration of agglutinins in the serum (1 to 20 to 1 to 200 dilution) or in the milk.

Br. abortus may be isolated from the uterine mucosa of cows which have aborted or from the gastro-intestinal tract and lungs of the fetus. Inoculate the infected materials on glycerol agar slants and incubate in an atmosphere containing 10 per cent CO_2 ; also inoculate a portion intra-

used for diagnosis. Serum with a titre up to 1 to 25 is considered suspicious. In infected pregnant animals the titre gradually rises before abortion to 1 to 200 or 1 to 1000. During the next six months it may fall to below 1 to 50. If the animal becomes a chronic carrier the titre may remain fairly high. The complement-fixation reaction may also be used.

Br. suis may be similarly isolated by culture or identified by serological tests with specimens from swine or other infected animals.

BACILLUS

The genus *Bacillus* is made up of organisms described as: "Rod-shaped bacteria, sometimes in chains. Aerobic. Non-motile or motile by means of peritrichous flagella. Endospores formed. Generally Gram-positive. Chemo-heterotrophic, oxidizing various organic compounds." The genus is a large one and most of its members are saprophytes. Eleven groups are now recognized within the genus and they contain 146 species. One species, *B. anthracis*, belonging to Group V or the *B. adhaerens* group, is highly pathogenic for animals and man. Another, *B. xerosporus*, found in Group VI or the *Aerobacillus* group, has sanitary significance because it may gain access to a water supply and give rise to a "false positive presumptive test" in water analysis inasmuch as it produces acid and gas from lactose and will grow in the lactose broth tubes commonly used in the bacteriological examination of water. The organisms of the genus are common in the environment, they grow readily on most media, they are aerobes and they form spores. Naturally, therefore, they constitute a very troublesome genus for they are common laboratory contaminants, easy to recognize, but hard to avoid. The "hay" bacillus, *B. subtilis*; the "potato" bacillus, *B. mesentericus*; *B. mycoides*; *B. megatherium*, the "cabbage" bacillus; and *B. cereus* are five of the more important species. In practice they are seldom differentiated, all being spoken of as "*subtilis*." All belong to Group I, the *B. subtilis* group. Aside from the fact that they are always getting in the way they are important since they may be mistaken for the anthrax bacillus.

Bacillus Anthracis

Agar Plate.—Colonies round, 2 to 3 mm. in diameter, raised, dull, opaque, grayish-white, with irregular borders, and uneven surface. Under microscope, colonies have a Medusa-head appearance. Membranous consistency, do not

emulsify easily; colony consists of characteristic parallel interlacing chains of
 cc.
 with uneven
 extensions,
 nec. Lique-

Broth.—No turbidity. Leaving the broth clear. If bling tufts of cotton arises a variable.

Resistance.—Spores ki" including the dry state,

Metabolism.—Aerobe,

Limits 12° to 44° C. 1.

improved by blood, serum or glucose. No hemolysin produced for human, cattle or horse blood.

Biochemical Reactions.—Acid, no gas in glucose, maltose, sucrose and salicin Indol —; nitrates reduced to nitrites. H_2S —, NH_4 =, methylene blue, reduced; catalase +. Litmus milk coagulated and decolorized; later peptonized; M. R. =; V. P. =.

Serology.—Precipitins in immune sera may be useful for identification. Anthrax antiserum produces cross reactions with many related species. Neither the agglutination nor the complement-fixation tests are of value for the diagnosis of infection.

Pathogenicity.—Naturally pathogenic to man, cattle, sheep (except Algerian), goats, pigs, and camels; rarely to carnivores. When injected subcutaneously into mice, guinea pigs, or rabbits they die in twelve to forty hours with a hemorrhagic local exudate, enlarged spleen, and bacilli in blood. Rats are less susceptible. Birds, except sparrows and young pigeons, cold-blooded animals, and fish are resistant.

Dissociation.—Growth, particularly in broth at a temperature of 42.5° C. for some days, causes the appearance of several variants, some have tough well-defined capsules, give rise on agar to the typical curled colonies, and are highly virulent; some have soft poorly defined capsules, form thin, shining colonies on agar, and are slightly virulent; others are non-capsulated, give rise to smooth, round convex, glistening, mucoid colonies on agar, and are entirely avirulent. Non-spore-forming variants may appear spontaneously in cultures incubated at the usual temperature, or in culture media containing antiseptics. Though there is a definite correlation between capsule formation and virulence, there may be none between spore formation and virulence.

Examination of Clinical Materials.—The source of the specimens used will depend, to some extent, on the type of infection. Collect pus or fluid from the local skin lesions (malignant pustule), blood in the septicemic stage of diseased animal, sputum in pulmonary infection (wool-sorter's disease) or spinal fluid in the case of a meningeal infection (rare). There is also an intestinal type of anthrax in which the bacillus might be found in the feces. It should be noted that in the septicemic stage of anthrax the bacilli, none showing spores, occur in large numbers in the blood and may be demonstrated by direct plating of a drop of blood. Animal products, such as shaving bristles, are sometimes examined for the presence of anthrax spores.

1. *Microscopic.*—Make film preparations with the infected material, stain by Gram's method and examine for the characteristic, large Gram-positive bacilli. Spores may be present only if the bacilli have been exposed to atmospheric oxygen. In blood or animal tissues the organisms are encapsulated. The long chains, so characteristically seen in culture, are seldom present in direct preparations.

2. **Cultural.**—Plant portions of specimen in nutrient broth and streak on agar plates. Incubate at 37° C. for twenty-four hours or more, and observe colonies. In case the material under study is a bacteriological mixture it is possible to kill off associated non-spore-bearing organisms by heating the broth in which the material has been planted to 60° C. for twenty minutes. This will not eliminate members of the "subtilis group" which might be present.

3. **Animal Inoculation.**—This is, in the case of anthrax, a very important diagnostic procedure. White mice, guinea pigs and rabbits are susceptible to anthrax infection, the mouse most so, the rabbit least. Inoculate one of these animals subcutaneously with a small amount of broth culture or suspension of agar growth. Blood may be injected directly or about 1 cc. of the heated (60° C., twenty minutes) saline washings of suspected shaving brush bristles. If anthrax bacilli are present, the animal will die with a fatal septicemia in from twelve to seventy-two hours. The organisms may then be isolated from the heart's blood, liver and spleen of the animal.

The diagnosis of anthrax is warranted if the specimen contains a Gram-positive, square-ended, chain-producing, spore-forming, non-motile bacillus which produces characteristic Medusa-head colonies on agar and, when injected subcutaneously into a white mouse or guinea pig, produces a fatal septicemia. Incidentally most of the species of the genus *Bacillus* are motile including all 34 members of the *B. subtilis* group in which are found the most important forms apt to cause trouble as contaminants around a laboratory. Furthermore these contaminants are slightly if at all pathogenic for guinea pigs.

CLOSTRIDIUM

Fifty-one species of Gram-positive, spore-bearing rods make up the genus *Clostridium* described as: "Anaerobes or microaerophiles, often parasitic. Rods frequently enlarged at sporulation, producing clostridium or plectridium forms." World War I gave impetus to the study of the large spore-bearing anaerobic bacilli because of the importance in that conflict of gas gangrene. *Cl. botulinum* causes botulism when its toxin is ingested. *Cl. tetani* has long been known as the etiological agent of tetanus or "lock-jaw." A variety of species participate in producing gas gangrene including *Cl. perfringens*, *Cl. sporogenes*, *Cl. noryi*, *Cl. septicum*, and *Cl. fallax*, the most frequently encountered. *Cl. chauvæi* causes blackleg, also known as symptomatic anthrax in cattle. A number of species are not pathogenic. Most of the species are found in soil and some are encountered in the intestinal tract. They appear to owe their pathogenicity, which is not insignificant, to the property many of them possess of exotoxin formation. Such species driven into the body by war wounds and accidents may produce serious illness. The toxin causing botulism is ingested and is one of the few toxins absorbed from the intestinal tract. There is still considerable confusion in the use of species names in this genus since many of the known species have been described under several different names.

Clostridium Tetani

Habitat —The tetanus bacillus is found in the intestinal tract of man and animals; also in cultivated soils.

as slender rods with

ance. Slightly motile, possessing

Stain readily with the ordinary aniline dyes, and are moderately Gram-positive,

stening, trans-

pearance. The

curled pro-

colonies.

translucent

beta zone of

of the tube. Unpleasant odor.

Cooked Meat Medium. Four Days at 37° C.—Good growth, with moderate turbidity, slight gas formation. No blackening or digestion of the meat, except after prolonged incubation.

Resistance.—Spores of *Cl. tetani* are very resistant to environmental influences, retaining their vitality for years in a dried condition. Resist boiling for fifteen to seventy minutes. All spores killed by exposure to dry heat, 160° C. for one hour, or to steam under pressure, 120° C. for twenty minutes. They also have high powers of resistance to certain antiseptics, withstanding the action of 5 per cent ph

wit

ho

sp

pH 7.4 to 7.8. Slight proteolytic powers; gelatin slowly liquefied, coagulated albumin not liquefied.

Biochemical Reactions.—No acid or gas formed in carbohydrate media. Indol positive; nitrates not reduced. Litmus milk, no change or slow precipitation of casein.

Examination of Clinical Materials.—In suspected cases of tetanus obtain pus or tissue scrapings from the wound, emulsify in a small amount of normal saline, and use for microscopic, cultural and toxicity tests. In clinical cases, cerebrospinal fluid may be drawn and tested for toxin.

1. Microscopic.—Make film preparations from the suspected material, stain, and examine for the characteristic "drum-stick" spores of *Cl. tetani*. If present in small numbers, they may be overlooked; or if non-virulent anaerobic and aerobic bacilli with round terminal spores are present, these may cause confusion, making this method of diagnosis of very little practical value.

2. **Cultural.**—Inoculate the specimen in cooked meat medium, and on blood agar and plain agar plates. In 1940 Brewer introduced thioglycollate media which greatly facilitate the culture of anaerobes.

Incubate at 37° C. for seventy-two hours under anaerobic conditions, preferably in a "Brown Jar," and examine for typical bacilli. If spores are present in the fluid-medium, heat the culture to between 75° and 80° C. for thirty minutes to kill any colonies. Pure cultures may also be obtained by inoculating the material into the water of condensation of an agar slant and then incubating the tube in an upright position. The tetanus bacilli produce an effuse, tenacious proteus-like growth over the surface of the slope. Subcultures from the edge of this fern-like growth into the condensation water of a fresh agar slant, will usually yield a pure culture after several transfers. After isolation study the organisms for cultural and biochemical characteristics. (See Table 70.)

TABLE 70.—CHARACTERISTICS OF THE MORE IMPORTANT SPECIES OF THE GENUS *CLOSTRIDIUM*
(Reed and Orr, courtesy of War Medicine)

Species	Milk	Dextrose	Maltose	Lactose	Salicin	Sucrose	Hydrogen sulfide	Gelatin liquefaction	Nitrate reduction	Indol	Milk agar digestion	Exotoxin
<i>Cl. uelchii</i> (<i>perfringens</i>)	Stormy	+	+	+	—	+	+	+	+	—	—	+
<i>Cl. butyricum</i> (group)	Stormy	+	+	+	+	+	+	+	+	—	—	—
<i>Cl. multifementans</i>	Stormy	+	+	+	+	+	—	+	+	—	—	—
<i>Cl. zoofatidum</i>	Stormy	+	+	+	+	—	+	+	+	—	—	—
<i>Cl. tertium</i>	Acid	+	+	+	+	+	+	—	+	—	—	—
<i>Cl. fallax</i>	Acid	+	+	+	+	+	+	—	+	—	—	+
<i>Cl. paraputrificum</i>	Acid	+	+	+	+	+	±	—	—	—	—	—
<i>Cl. carnis</i>	Acid	+	+	+	+	+	—	—	—	—	—	+
<i>Cl. chauvæi</i>	Acid	+	+	+	—	+	+	+	+	—	—	+
<i>Cl. septicum</i>	Acid	+	+	+	+	—	+	+	+	—	—	+
<i>Cl. sphenoides</i>	Acid	+	+	+	+	—	+	—	—	+	—	—
<i>Cl. novus</i> (<i>adematens</i>)	Digested	+	+	—	—	—	+	+	—	—	+	+
											or	
<i>Cl. bifementans</i>	Digested	+	+	—	—	—	+	+	—	+	+	—
<i>Cl. sordellii</i>	Digested	+	+	—	—	—	+	+	—	+	+	+
<i>Cl. sporogenes</i>	Digested	+	+	—	—	—	+	+	—	—	+	—
<i>Cl. histolyticum</i>	Digested	—	—	—	—	—	+	+	—	—	+	+
<i>Cl. tetanomorphum</i>	No change	+	+	—	—	—	±	—	—	±	—	—
<i>Cl. difficile</i>	No change	+	—	—	+	—	±	±	—	—	—	+
<i>Cl. captovalis</i>	No change	+	—	—	—	—	±	±	—	+	—	—
<i>Cl. cochlearium</i>	No change	—	—	—	—	—	—	—	—	—	—	—
<i>Cl. tetani</i>	No change	—	—	—	—	—	+	±	—	—	—	+

3. **Animal Inoculation.**—Mix a portion of the original material, or of the heated culture, with sterile emery dust and inject it subcutaneously into the thigh of a guinea pig. Results are more satisfactory when a broth suspension of a pure culture is used. A control animal protected by an intraperitoneal inoculation of tetanus antitoxin should receive a similar injection at the same time. If *Cl. tetani* is present, the unprotected animal will develop tetanus and die within one to four days.

Demonstration of Toxin in Broth Cultures.—Inject subcutaneously 0.5 cc. of filtrate of a ten-day broth culture into each of two mice or guinea pigs, one animal of which has been given a prophylactic dose of antitoxin. Similar toxicity tests may be made by injecting animals with spinal fluid from cases of tetanus.

Clostridium Botulinum

Habitat.—Primarily a saprophyte of soil origin, but may be found occasionally in the intestinal tract of domesticated animals.

Morphology.—Large Gram-positive rods 4 to 6 μ long and 0.9 to 1.2 μ wide, . . . Spores
 . . . Spores
 . . . motile by

Agar Plate. Four Days at 37° C.—Flat, irregular, grayish-yellow, filamentous definite,
 ze, thin
 Abun-

Gelatin Stab. Ten Days at 37° C.—Liquefied and blackened.

Blood Agar Plate (Horse). Three Days at 37° C.—Irregularly round, 2 to 3 mm. in diameter, umbonate colonies, with smooth center and fimbriate periphery. Alpha type hemolysis.

Resistance.—The bacilli without spores are readily killed by heat and chemicals. Spores with 100° C. for . . .
Metabolis
 or slightly
 Hemolysin
 proteolytic;
 impeded; b-

Biochemical Reactions.—Two biochemical saccharolytic. Proteolytic includes serology. Saccharolytic includes some strains of type . . .
 and B. Destroys levulose and maltose are . . .

Serology.—At present there are recognized five types of *C. botulinum* which differ from one another in the immunological specificity of their toxins. Types

one form of forage poison subcutaneously in mice, proves fatal in one to four days. American outbreaks is 67 per cent.

Examination of Clinical Materials.—Botulism may be associated with meat or meat products, fruits, vegetables, canned goods, and various pickled and preserved foodstuffs. (Refer to Chapter XXVII on Bacterial Food Poisoning, for methods of examination.)

ORGANISMS ASSOCIATED WITH GAS GANGRENE

there are fields and gardens fertilized with animal manure. It is clear that

such wounds contain aerobic organisms in addition to anaerobes and gas gangrene may not be the first or only untoward result of such trauma. Such wounds are, of course, a possible source for the introduction of tetanus bacilli or spores and must be given treatment designed to prevent the development of that disease. The anaerobic organisms associated with gas gangrene may be divided on the basis of pathogenicity into three groups:

1. Of prime pathogenicity:

(a) For man:

- (1) *Cl. perfringens* (*Cl. welchii*).
- (2) *Cl. septicum* (*Vibrio septique*).
- (3) *Cl. novyi* (*Cl. œdematiens*).
- (4) *Cl. bifermentans* (*Bacillus sordelli*).

(b) For animals:

- (1) *Cl. chauvæi*.

2. Of lesser pathogenicity for man:

- (1) *Cl. histolyticum*.
- (2) *Cl. fallax*.

3. Themselves non-pathogenic but contributing nevertheless to the gaseous gangrene syndrome:

- (1) *Cl. sporogenes*.
- (2) *Cl. zoofætidum*.
- (3) *Cl. lentoputrescens* (*Cl. putrificum*).
- (4) *Cl. tertium* (*Bacillus tertius*), and others.

On the basis of their biochemical reactions the anaerobes associated in the production of gas gangrene may be separated into a proteolytic and a saccharolytic group. These properties are not strictly demarcated for most members have some properties of the other group. Classification is, however, made according to predominating activity. Most of the pathogenic group are saccharolytic and, with the exception of *Cl. histolyticum*, most of the proteolytic organisms are non-pathogenic. These proteolytic organisms are saprophytes, have no power of invading the tissues, and if present in a wound, in the absence of saccharolytic anaerobes, usually do not interfere with the healing of the wound. They do complicate wounds by their intense proteolytic action and thus they contribute to the vicious cycle set up in a progressing, untreated gaseous gangrene. The anaerobic flora in gas gangrene is often mixed with *Cl. perfringens* occurring most frequently. This species is also most common when only a single species of anaerobe is present.

Clostridium Perfringens

Habitat.—The Welch bacillus, often called *Cl. welchii*, is found in the intestina

hort chains. Capsules formed in the animal terminal, being formed only in alkaline sugar-free media. Wide range of pleomorphism in old cultures. Spores are large, oval, and central or sub-

Agar Plate. Forty-eight Hours at 37° C.—Circular colonies from 0.5 to 1 mm. in diameter, moist, slightly domed, pearl gray, opaque, with a smooth surface and an entire edge.

Deep Glucose Agar Shake.—Lenticular colonies with an entire edge; abundant gas formation.

Blood Agar Plate (Horse). Forty-eight Hours at 37° C.—Round domed colonies from 1 to 1.5 mm. in diameter, grayish-white with a smooth glistening surface, and an entire edge. Zone of beta hemolysis from 2 to 4 mm. surrounding each colony.

Gelatin. Two Days at 37° C.—Liquefied and blackened.

Broth. Four Days at 37° C.—Moderate turbidity with gas formation, and white powdery sediment.

Cooked Meat Medium. Fifteen Days at 37° C.—Rapid growth with gas formation.

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um
by

Biochemical.—Acid and gas in dextrose, levulose, galactose, mannose, maltose, raffinose and inositol, variable in mulin and Indol —; M. R. +; V. P. —; nitrates =. Little profuse gas formation (stormy fermentation). In their ability to ferment inulin and glycerol

polysaccharide known as hyaluronic acid widely distributed in connective tissue thus increasing the permeability of the tissues and facilitating extension of the gaseous gangrene process

Serology.—Agglut

stained.

Dissociation.—Pure smooth colonies may give rise to mucoid and rough granular

Short rod, granular variant colonies may be more toxic than the parent strain.

Demonstration of Anaerobes in Wounds.—"The case mortality from wound infections due to the gas gangrene organisms remains obstinately in the region of 30 to 60 per cent. In the early stages of infection when the tissues are still capable of recovery the clinical changes are so slight that, even with considerable experience, the surgeon can seldom form a definite opinion as to the presence of an anaerobic infection." Thus McClean and co-workers present the need for an early diagnosis. They have suggested (1913) that search for hyaluronidases or lecithinase in the early hours of the wound history may be an approach to "Early Diagnosis of Wound Infection." As usually carried out anaerobes in wounds are revealed by microscopic and cultural examination and animal inoculation of pus, serous exudates or infected tissue from wounds, fluid from hemorrhagic blebs or blood.

1. *Microscopic.*—Make film preparations of the wound specimens, stain by Gram's method and by one of the capsule stains; also make hanging

drop preparations. The presence of Gram-positive rods suggests the presence of anaerobes but does not warrant a diagnosis of "gas gangrene." The finding of large Gram-positive, non-motile, capsulated rods is presumptive evidence that *Cl. perfringens* may be present, while the presence of Gram-positive motile rods indicates the presence of associated wound organisms.

2. **Cultural.**—The infected materials may be used to inoculate duplicate sets of culture media, including litmus milk, Robertson's medium, infusion broth, infusion agar plates and blood agar plates. All media used for the isolation of anaerobes should be boiled for ten minutes and cooled rapidly just before the inoculations. The cultures should be incubated at 37° C., one set kept in an ordinary incubator and the other under anaerobic conditions, preferably in a "Brown Jar." If the latter apparatus is not available, as for example under field conditions, a state of partial anaerobiosis can be maintained by covering all the fluid media with sterile liquid petrolatum or melted vaseline and by using deep dextrose agar shake cultures instead of the plates. Brewer's thioglycollate medium may be used in which case attention to mechanical anaerobiosis is unnecessary.

The identification of the spore-bearing anaerobes is somewhat more difficult than in the case of the aerobic and facultatively anaerobic bacteria and can hardly be adequately dealt with here.

A tentative diagnosis of *Cl. perfringens* can be made upon observing its typical reaction in indicator milk. Within twenty-four hours, if *Cl. perfringens* is present, there will occur an acid clot torn by gas, the so-called "stormy fermentation." Stained smears reveal the stout Gram-positive Welch bacillus. If this reaction does not occur within the time specified, it may be assumed that some other species of anaerobic bacillus is present. As soon as a characteristic clot has appeared, usually within eight hours, inoculate a second paraffined milk tube, heat at 60° C. for forty-five minutes (to kill vegetative forms of other organisms) incubate twenty-four hours and from the resultant growth inoculate a series of agar shake tubes. Observe for growth of characteristic opaque lenticular colonies.

As *Cl. septicum* and *Cl. sporogenes* produce spores in Robertson's medium under anaerobic conditions within twelve hours and *Cl. perfringens* does not, these organisms may be partially separated as follows: After twelve hours incubation, heat a portion of the anaerobic cultures in Robertson's medium to 80° C. for twenty minutes to destroy the vegetative forms of *Cl. perfringens* and any aerobic organisms still alive and then make transfers to plates of serum or blood agar. After anaerobic incubation, select single colonies for transfer to broth and continue this procedure until pure cultures are obtained.

When dealing with mixed cultures, the growth of *Cl. perfringens* can be concentrated by anaerobic incubation for three to six hours in a tube of glucose broth. *Cl. septicum*, the *Vibrio septique* of Pasteur, can be similarly concentrated in salicin broth or *Cl. sporogenes* in a medium of tap water containing coagulated egg albumen. Certain of the pathogenic species may be further separated by inoculation into animals.

3. **Animal Inoculation.**—If it is suspected that *Cl. perfringens* is present a rapid tentative diagnosis may be made as follows: Inoculate a suspension of the infected material or culture intravenously into a normal rabbit. Five minutes later kill the animal and place the body in an incubator at

37° C. for twelve to eighteen hours. The animal becomes distended with gas, bubbles of which may be found along the vessels and in the organs, especially in the liver. *Cl. perfringens* may be found in smears and cultures from these lesions. However, this method is not infallible, as occasionally there may be postmortem invasion by anaerobic organisms already present in the animal. We have also found that a similar condition in the rabbit can be produced by injecting highly aerogenic coliform organisms and have encountered one case of suspected "Welch bacillus infection" in which the organisms found postmortem in the liver were coliform and not anaerobic bacilli at all.

The final confirmatory test applicable to *Cl. welchii*, *Cl. oedematis* or *Cl. oedematis-maligni*, as well as to *Cl. tetani*, consists in the inoculation of pure cultures of the anaerobes into normal guinea pigs, using as controls animals which have been protected by an inoculation of antitoxic serum specific against one or more of the suspected pathogenic organisms.

MALLEOMYCES

Two species, both pathogenic, constitute the genus *Malleomyces*. Organisms of the genus are described as: "Short rods, with rounded ends, sometimes forming threads and showing a tendency toward branching. Motile or non-motile. Gram-negative. Tendency to bipolar staining. Milk slowly coagulated. Gelatin may be liquefied. Specialized for parasitic life. Grow well on blood serum and other body fluid media." The most important species is *Malleomyces mallei*, the cause of glanders, a disease of horses, transmissible to man. *Mal. pseudomallei*, Whitmore's bacillus, causes melioidosis in rats, guinea pigs, rabbits and in man in India, Federated Malay States and Indo-China. The vagaries of bacterial taxonomy are nowhere better illustrated than in the case of the organism causing glanders. At present known as *Mal. mallei* it has been called *Bacillus mallei*, *Corynebacterium mallei*, *Mycobacterium mallei*, *Bacterium mallei*, *Pfeifferella mallei*, *Brucella mallei*, *Loefflerella mallei*, and in the previous edition of this text it was discussed under the name *Actinobacillus mallei*. A factor certainly in this remarkable record is the nature of the organism itself. As will be seen its characteristics suggest similarity to a number of other well-known bacteria.

Key to the Species of the Genus *Malleomyces*

I. Carbohydrates not fermented. Honey-like colonies on potato. Glycerol agar colonies slimy or tenacious, translucent. Non-motile.

1. *Malleomyces mallei*.

II. Carbohydrates fermented. Profuse creamy growth on potato. Glycerol agar colonies iridescent, becoming corrugated. Motile.

2. *Malleomyces pseudomallei*.

Malleomyces Mallei

Habitat.—A strict parasite, found chiefly in equines; occasionally in man.

Morphology.—Slender, non-motile, non-sporulating, non-capsulated rod, with rounded ends, about 0.3 to 0.5 μ wide, and 1.5 to 3 μ long. The rods are usually straight, but slightly curved forms may be seen, arranged singly, in pairs or in small groups in the tissues. In culture there may be considerable pleomorphism,

ing irregular bizarre
or false branching.
ules which may be
methylene blue;

Gram-negative, non-acid-fast.

Agar

1 mm. in

twenty-f

in color, more opaque, and may have a finely granular surface. Butyrous
in consistency and easily emulsifiable

growing growth on potato acquires a greenish-yellow tint.

Broth.—Moderate growth with turbidity; thin surface pellicle, and slimy or
ropy tenacious sediment.

Resistance.—Destroyed by moist heat at 55° C. in ten minutes or by ordinary
antiseptics in from ten to fifteen minutes. Dried cultures may live for three or
four weeks.

temperature 37° C.;
1. Growth develops

Some strains may
alk usually acidified
itrates not reduced.

Some strains may
alk usually acidified
itrates not reduced.
Precipitin tests are less satis-
test).

tem: In addition to the
and lesions in the liver,
sh-white nodules. Bacilli

ACTINOBACILLUS

Three species at present constitute the genus *Actinobacillus* in which
Mal. mallei was formerly placed. All three species are found in lesions of
actinobacillosis in bovines, a disease which resembles actinomycosis.
Actinobacillus species do not commonly attack man. The organisms of
this genus are described as: "Medium-sized aerobic Gram-negative rods
which frequently show much pleomorphism. Coccus-like forms frequent.
Acid but not gas usually produced from carbohydrates. Grow best, espe-
cially when freshly isolated, under increased CO₂ tension. Pathogenic for
animals; some species occasionally attack man. The outstanding character-
istic of the group is the tendency to form aggregates in tissues or culture
which resemble the so-called sulfur granules of actinomycosis."

Actinobacillus Lignieresii

Actinobacillus lignieresii is the cause of actinobacillosis in bovines, a disease which resembles closely actinomycosis. The organisms attack mainly the soft tissues and rarely infiltrate the bone. The lymphatic glands in the region of the mouth and neck are areas particularly affected (woody tongue). In young cultures, it is a small, Gram-negative, non-motile bacillus, measuring about 1.5μ in length and 0.1μ in width. In lesions in the animal body, small opaque granules are present in the purulent discharge. The centers of these granules are made up of masses of Gram-negative clubs. Growth occurs readily on nutrient agar, forming small, circular, semi-transparent, bluish-gray colonies, with a smooth surface and an entire edge, attaining a diameter of 1.5 mm. in twenty-four hours at 37°C . The agar colonies are composed of typical short bacilli, but in glucose agar shakes, the colonies consist of long, tangled, unbranched filaments. In serum broth streptobacillary forms appear. Growth is poor on gelatin and on Loeffler's serum, no liquefaction occurring. On acid potato there is no growth, but on alkaline potato a slight yellowish growth. Acid is formed in dextrose and lactose. Litmus milk is acidified, but not coagulated. Indol —; nitrates not reduced. The organism is not resistant, killed by heat at 62°C . in ten minutes, succumbs readily to drying, requires frequent transplanting. Experimentally, cattle, pigs, guinea pigs, cats, dogs, and rabbits are susceptible to infection. Large doses injected intraperitoneally into guinea pigs cause death in eighteen to twenty-four hours, smaller doses ($\frac{1}{4}$ of an agar slant culture) injected intraperitoneally into male guinea pigs produce the typical Straus reaction. Agglutinins develop in infected animals and may be useful in diagnosis. No cases of human infection have been reported.

Examination of Clinical Materials.—Refer to Chapter XLIII under Special Veterinary Laboratory Methods for information on the examination of specimens from glanders and actinobacillosis.

ACTINOMYCES

The genus *Actinomyces* of the family *Actinomycetaceae*, as at present constituted, includes 62 species described as follows: "Organisms growing in the form of a much branched mycelium, which may break up into segments that function as conidia. Sometimes parasitic, with clubbed ends of radiating threads conspicuous in lesions in the animal body. Some species are microaerophilic or anaerobic. Non-motile." Eight species are described as animal parasites, nine species are said to be plant pathogens and the remaining members of the genus are nonpathogenic saprophytes of soil origin.

Actinomycosis was first recognized as a specific parasitic disease by Billinger in 1877. At his suggestion the botanist, Hatz (1878) studied the disease and called the etiological agent *Actinomyces viscosus* on account of the ray-like structure of its growth in the test tube. The early work was carried out without benefit of modern reference and it would seem probable that much of the taxonomic confusion in this group arises from a liberal substitution of parasitic and saprophytic forms. The *Actinomyces* is a strictly anaerobic and non-motile that growth requires a reduced oxygen

"domestication." It is the principal cause of actinomycosis, whether in man or in animal. *Act. hominis* is aerobic and while it has been isolated

is of at least eight different types clinically, each with its own etiology. According to Brumpt actinomycotic mycetoma is caused by the ray-fungus *Act. bovis*, whereas Vincent's white mycetoma is caused by *Act. maduræ*. This latter form is not so serious a disease as the former and *Act. maduræ*, anaerobe, is not pathogenic for animals.

Actinomyces Bovis

Habitat.—Strictly parasitic, occurring chiefly in lesions of actinomycosis in cattle; occasionally in man.

Morphology.—On culture media rods 3 to 4 μ long and 0.6 μ wide, long thin continuous forms.

In old granules, the central mycelium frequently breaks up into small swellings, staining Gram-negatively. In old granules, the central mycelium frequently breaks up into small swellings, staining Gram-negatively. In old granules, the central mycelium frequently breaks up into small swellings, staining Gram-negatively. In old granules, the central mycelium frequently breaks up into small swellings, staining Gram-negatively.

granule containing a central mycelium and tissues as granule contained by a radial swelling, staining Gram-positive

swellings, staining Gram-positive. In old granules, the central mycelium frequently breaks up into small swellings, staining Gram-positive. In old granules, the central mycelium frequently breaks up into small swellings, staining Gram-positive.

Broth.—Growth poor; improved by the addition of glucose, medium clear, no surface pellicle. At the bottom, compact, irregular masses that fail to disintegrate

swellings, staining Gram-positive. In old granules, the central mycelium frequently breaks up into small swellings, staining Gram-positive. In old granules, the central mycelium frequently breaks up into small swellings, staining Gram-positive.

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may be retained for weeks in the refrigerator. Killed by moist heat at 60° C. in ten minutes.

Metabolism.—Anaerobic; temperature 37° C., minimum non-chromogenic. Growth on glucose or glucose.

Optimum temperature 37° C., minimum non-chromogenic. Growth on glucose or glucose.

cultures into rabbits or guinea pigs, may give rise, in several weeks or months, to non-progressive tumor-like lesions, from which the organisms can be occasionally recovered

Examination of Clinical Material.—1. Microscopic.—Pus from the suspected lesions should be spread thinly in a sterile Petri dish, or diluted

with physiological saline, and thoroughly searched for the presence of the characteristic granules. Since the organisms are usually confined to the granules, these must be found if microscopical examination of pus is to assist in detecting the infection. The granules should be examined: (a) unstained under a cover glass without crushing, using a subdued light, for the detection of a peripheral fringe of club-shaped forms, and the characteristic rough or corrugated surface of the granule, and (b) examined after crushing and staining by Gram's method, for the presence of a mycelium of Gram-positive filaments, surrounded by radially arranged Gram-negative pear-shaped enlargements.

[illegible]

MYCOBACTERIUM

Thirteen species of acid-fast bacteria at present constitute the genus *Mycobacterium* which is defined as follows: "Slender rods which are stained with difficulty, but when once stained are acid-fast. Cells sometimes show swollen, clavate or cuneate forms, and occasionally even branched forms. Growth on media slow for most species. Aerobic. Several species pathogenic to animals."

Key to the Species of Genus *Mycobacterium*

- I. Parasites in warm-blooded animals, grow slowly on all media
- A. Grow slowly on glycerol agar in atmospheric air, experimentally infect guinea pigs and fowls.
1. Experimentally produces generalized tuberculosis in guinea pigs but not in fowls. Growth enhanced by addition of glycerol to media. Colonies generally pale yellow to orange pigmentation on agar.
 - (1) *Mycobacterium tuberculosis* var. *hominis*.
 2. Experimentally produces generalized tuberculosis in guinea pigs and rabbits but not in fowls.* Growth not enhanced by addition of glycerol to media. Never pigmented.
 - (2) *Mycobacterium tuberculosis* var. *bovis*.
 3. Experimentally produces generalized tuberculosis in fowls but not in guinea pigs.*
 - (3) *Mycobacterium avium*.
- B. Grows on glycerol agar only when extracts of, or heat-killed acid-fast bacilli added. Experimentally fails to infect guinea pigs or fowls.
- (4) *Mycobacterium paratuberculosis*.
- C. Grows slowly, if at all, on culture media and only in the presence of high concentration of CO₂ and O₂. Experimentally fails to infect guinea pigs or fowls.
- (5) *Mycobacterium leprae*.

* Within the limits of the dosages used in standard procedure.

II. Saprophytes or parasites on cold-blooded animals; grow rapidly on most media.

A. Fails to survive 60°C . for one hour.

1. Fails to grow at 47°C .

(a) Unable to utilize sorbitol.

(6) *Mycobacterium piscium*.

(7) *Mycobacterium marinum*.

(8) *Mycobacterium ranze*.

(9) *Mycobacterium thamnophaeos*.

(b) Utilizes sorbitol.

(10) *Mycobacterium chelonci*.

(11) *Mycobacterium* spp.

2. Grows at 47°C .

(12) *Mycobacterium lacticola*.

B. Survives 60°C . for one hour; grows at 47°C .

(13) *Mycobacterium phlei*.

Mycobacterium Tuberculosis var. *Hominis*

Habitat.—Strict parasite causing tuberculosis in man, pigs, monkeys, dogs and

5 to 46 μ in length, and rounded ends. May show branching.

ing. Non-motile, non-sporing and non-capsulated. Do not stain with ordinary

Stain best with hot carbolfuchsin, resist

1 per cent H_2SO_4 for at least ten minutes;

evenly or irregularly, showing granular,

beaded, or banded forms. Cultures may show non-acid-fast and clubbed forms.

The organisms from the animal body appear larger than those from cultures.

Agar Plate, Four Weeks at 37°C .—No growth.

Glycerol Agar, Four Weeks at 37°C .—Colonies minute, crumb-like, irregular, moist, whitish-yellow, later brownish, ridged, becoming dry.

—Flat, spreading, crumpled to rugose

it, discrete or confluent, slightly raised,

surface.

7°C .—Colonies 1 to 3 mm, yellowish

be low domed with a central depression

are indented.

Glycerol Potato, Four Weeks at 37°C .—Thick, raised, confluent growth, creamy

or yellow in color.

four hours.

Metabolism—Aerobic, no growth under strictly anaerobic conditions. Growth

occurs best in an atmosphere of

ary

or

Evidence

, sucrose,

2. *Endamoeba coli* (Grassi, 1879) Hickson, 1909.—This organism has three stages also in its life cycle, but it is non-pathogenic for man or animals.

(a) *Trophozoites*.—Size, 15 to 50 μ in diameter, with an average of 20 to 30 μ . In fresh warm feces, the organisms are sluggish in their movements. Although clear ectoplasm can be differentiated occasionally, the organism is mainly endoplasm containing vacuoles, bacteria and fecal debris. Red blood corpuscles may be ingested if they are present in the stool. The nucleus is generally visible as a ring of granules with an eccentric karyosome, because of the low refractivity of the endoplasm (Fig. 64, Nos. 1 and 2).

In preparations stained with hematoxylin, the nucleus differs from that of *E. histolytica* by having a thicker nuclear membrane, larger and heavier staining granules of peripheral chromatin and a larger karyosome in an eccentric position (Fig. 64, No. 1). The stained cytoplasm reveals the presence of the varied particles that are ingested.

The precystic stages are formed in a manner similar to those of *E. histolytica* but the precysts (Fig. 64, No. 3) and cysts are larger in average diameter. The nuclear structure is similar to that of the trophozoites.

(b) *Cysts*.—Size, 10 to 30 μ in diameter, with an average of 15 to 18 μ . The cysts are spherical containing 1, 2, 4, or 8 nuclei (Fig. 64, Nos. 4 to 7). The first cysts are uninucleate, but three successive divisions occur to produce the 8 nuclei of the typical mature cysts. Supernucleate cysts with 16, 32 or more nuclei occur also.

The glycogen vacuole is much larger in the early cystic development and the chromatoid bodies are irregular or pointed in shape and often filamentous. The chromatoid material and the vacuole usually disappear during the early nuclear divisions, leaving only the nuclei and clear cytoplasm in the mature cyst. Uninucleate or binucleate cysts of this amoeba are difficult to distinguish from similar cystic stages of *E. histolytica*, but the presence of 8 nuclei in the mature cyst is diagnostic.

(c) *Life Cycle*.—In many respects, the life cycle of *E. coli* is similar to that of *E. histolytica*. Mature cysts are ingested with food or drink contaminated with feces. Excystation of the cysts takes place in the small or large intestine to liberate a metacystic amoeba containing 8 nuclei or in some cases 4 to 7 nuclei, because of occasional nuclear degeneration. Partition of the cytoplasm of the metacystic amoeba takes place producing 4 to 8 amoebulae. These organisms grow and proceed to multiply by binary fission, forming typical trophozoites of *E. coli*. These trophozoites can encyst again when certain conditions obtain to form the infective stages by the characteristic cystic development.

The incidence of *E. coli* is generally higher than *E. histolytica*, reaching to 50 per cent in tropical areas. The significance of this fact must be emphasized because of the rôle played by this organism in arriving at a specific diagnosis of *E. histolytica*.

3. *Endamoeba gingivalis* (Gros, 1849) Smith and Barrett, 1915.—(a) *Trophozoite*.—Size, 5 to 35 μ (Fig. 63, No. 12). Examination of material from carious teeth or abscesses in the oral and pharyngeal regions frequently reveals an amoeba which in a number of ways resembles *E. histolytica*. However, this amoeba is not pathogenic but only a saprophytic organism which lives in the mouth. The ectoplasm and endoplasm are clearly dif-

ferentiated and numerous food vacuoles may contain degenerating leucocytes, tissue cells and bacteria. The nucleus contains a karyosome which is central or eccentric, and peripheral chromatin composed of granules so closely packed that they resemble a ring. Division takes place by binary fission and no cystic stages are known.

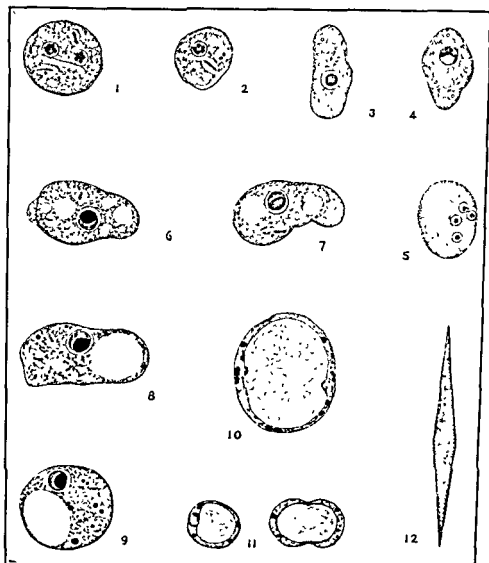


FIG. 65.—Other intestinal amebae of man ($\times 2000$). 1 and 2, Trophozoites of *Dientamoeba fragilis*; 3 and 4, trophozoites of *Endolimax nana*; 5, cyst of *E. nana*; 6 and 7, trophozoites of *Iodamoeba bütschlii*; 8 and 9, cysts of *I. bütschlii*; 10 and 11, *Blastocystis hominis*; 12, Charcot-Leyden crystal. (Heidenhain's iron-alum hematoxylin stain) (Original)

1. *Dientamoeba fragilis* Jepps and Dobell, 1918.—(a) *Trophozoites*.—Size, 3 to 12 μ . Although this unusual organism has been classified and named as an ameba, careful morphological evidence has been presented to show affinities with the flagellates (Dobell, C., *Parasit.* 32, 417, 1910). *Dientamoeba* possesses two nuclei of identical size and structure which in favorable stained specimens are seen to be connected with a thin fibril or "centrodesmus" (Fig. 65, No. 1). A certain percentage of the organisms

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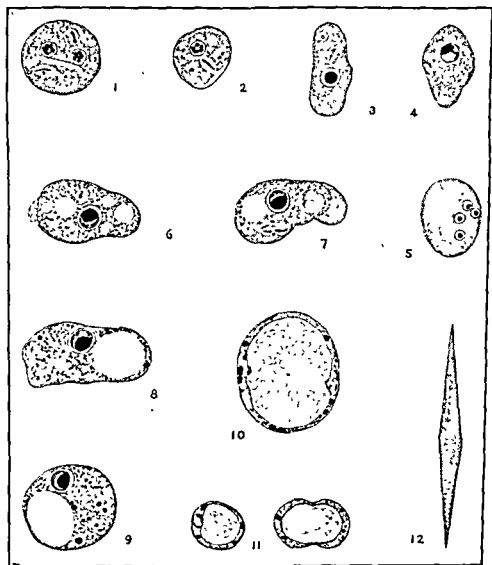


FIG. 65.—Other intestinal amoebae of man ($\times 2000$). 1 and 2, Trophozoites of *Dientamoeba fragilis*; 3 and 4, trophozoites of *Endolimax nana*; 5, cyst of *E. nana*; 6 and 7, trophozoites of *Iodamoeba bütschlii*; 8 and 9, cysts of *I. bütschlii*; 10 and 11, *Blastocystis hominis*; 12, Charcot-Leyden crystal. (Heidenhain's iron-alum hematoxylin stain) (Original)

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contain only one nucleus (Fig. 65, No. 2). Each nucleus is vesicular containing a central mass usually composed of 6 granules or chromosomes (according to Dobell). One of the granules is larger and stains more deeply than the others. The cytoplasm is differentiated between ectoplasm and endoplasm, with the endoplasm containing numerous food particles and vacuoles. No cysts are known for this species and the method of transmission is unknown.

associated intestinal disturbances.

5. *Endolimax nana* (Wenyon and O'Connor, 1917) Brug, 1918.—(a) *Trophozoite*.—Size, 6 to 12 μ in diameter. This organism is a small amoeba that has few identifying characters in the living condition. In stained preparations, the nucleus has a characteristic large karyosome which assumes a variety of shapes in freshly fixed and stained preparations but may be rounded in preparations made from older fecal samples (Fig. 65, Nos. 3 and 4).

(b) *Cysts*.—Size, 8 to 10 μ by 7 to 8 μ . The cysts are typically oval (Fig. 65, No. 5) containing 1, 2, or 4 nuclei similar to those of the trophozoites. No cytoplasmic inclusions are present.

6. *Iodamoeba bütschlii* (v. Prowazek, 1911) Dobell, 1919.—(a) *Trophozoite*.—Size, 6 to 20 μ in diameter. Living organisms in a freshly passed stool move sluggishly with the extrusion of clear ectoplasmic pseudopodia. The nucleus is not usually visible and the cytoplasm contains a variety of food particles. In stained organisms, the morphology of the nucleus distinguishes this amoeba from other intestinal species (Fig. 65, Nos. 6 and 7). The most conspicuous structure is the relatively large karyosome which may be rounded or irregular in shape. A layer of chromatic granules, which stain less intensely, is dispersed between the karyosome and the nuclear membrane. These granules aid in distinguishing small organisms of this species from *Endolimax nana*.

(b) *Cyst*.—Size, 7 to 13 μ . Although the size of these cysts is very close to that of *E. histolytica*, the oval or irregular shape and the presence of only one nucleus is diagnostic (Fig. 65, Nos. 8 and 9). Staining of the cysts in a fresh preparation with iodine reveals a deeply staining brown mass of glycogen which led to the original name of "iodine cysts" for these stages of *Iodamoeba*. The mass does not stain in hematoxylin preparations, but appears as a vacuole in the cytoplasm. The structure of the nucleus in stained cysts shows a karyosome that is more eccentric, and chromatic granules that are frequently aligned in the form of a crescent near the karyosome. The cysts are the infective stages but little is known about the life cycle.

7. *Giardia lamblia* Stiles, 1915.—(a) *Trophozoite*.—Size, 10 to 18 μ by 6 to 11 μ . This organism, which lives in the small intestine of man, is bilaterally symmetrical with two nuclei and four pairs of flagella (Fig. 66, No. 1). The anterior end is marked by a concave ventral sucking disc but there is no oral opening (Fig. 66, No. 2). The posterior end tapers to form a tail from which a pair of flagella extend. The disposition of skeletal fibers and axostyles can be seen in Figure 66, No. 1. No food particles

can be seen in the body; the organisms apparently absorb dissolved nutritive material by osmosis.

(b) *Cyst* (Fig. 66, No. 3).—Size, 8 to 14 μ by 6 to 10 μ . The trophozoites encyst as they pass through the large intestine. The resultant cysts passed off in the feces are the resistant and infective stages of the parasite. Their shape is ovoid and the cyst wall is relatively thick. When stained, 2, 4 or more nuclei can be seen. The fibers and even flagella remain differentiated in the cyst. Since the trophozoites live in the small intestine, the cysts are more frequently found in the stools and occasionally in great numbers. This organism is very common in children with an incidence up to 50 per cent; in adults the incidence is 2 to 15 per cent.

8. *Chilomastix mesnili* Wenyon, 1910.—(a) *Trophozoite* (Fig. 66, No. 4).—Size, 6 to 20 μ by 3 to 4 μ . This organism is one of the common intestinal flagellates with an incidence of 1 to 11 per cent. It differs from the trichomonads by having a prominent cytostome containing a flagellum, no undu-

end and fibrils can be detected along the margin of the cytostome in stained specimens. The movement of this flagellate is slow and deliberate when compared with the jerky motion of trichomonads.

(b) *Cyst* (Fig. 66, No. 5).—Size, 6 to 10 μ in length by 4.5 to 6 μ in width. The characteristic pear-shape is distinctive. Stained specimens show a single nucleus. Binucleate cysts are very rare and abnormal when they occur. The cytostome, flagella and fibrils can be detected and there is a cap on the anterior end.

9. *Enteromonas hominis* (da Fonseca, 1915) (Synonym *Tricercomonas intestinalis* Wenyon and O'Connor, 1917).—(a) *Trophozoite* (Fig. 66, No. 6).—Size, 4 to 10 μ by 3 to 6 μ . This organism has a characteristic ovoid shape that is flat on one side. There are three anterior flagella and a fourth flagellum which trails from a fibril extending posteriorly through the cytoplasm from the anterior blepharoplast. The nucleus is vesicular with a large central karyosome.

(b) *Cyst* (Fig. 66, No. 7).—Size, 6 to 8 μ by 3 to 4 μ . Their shape is elongately ovoid and 1, 2, or 4 nuclei may be present. The incidence of this organism may be as high as 7 per cent in tropical countries.

10. *Trichomonas vaginalis* Donné, 1836.—(a) *Trophozoites* (Fig. 66, No. 10).—Size, 7 to 36 μ in length. Incidence—3 to 50 per cent. When seen swimming freely in a fresh preparation from vaginal secretion or from young cultures, the body is spindle-shaped, but when among cellular debris, the body is extremely plastic and assumes a great variety of shapes as it burrows between particles. Four flagella arise from a blepharoplast in the anterior end. A fifth flagellum extends along the margin of the characteristic undulating membrane, ending with the membrane posteriorly one-third to two-thirds of the body length. The disposition of the fibrils and chromatic granules can be seen in Figure 66, No. 10. The nucleus is vesicular and is found in the anterior end near the blepharoplast. A skeletal structure or axostyle arises at the anterior end, passing through the center of the body and protruding posteriorly. The cytostome is at the anterior end and ventral to the origin of the undulating membrane. The parabasal

apparatus (Wenrich, 1939) is to be found near the nucleus but special staining procedures are required to detect it. No cysts are known.

11. *Trichomonas hominis* Davaine, 1860.—(a) *Trophozoites* (Fig. 66, No. 11).—Size, 7 to 20 μ . This organism lives in the large intestine and is

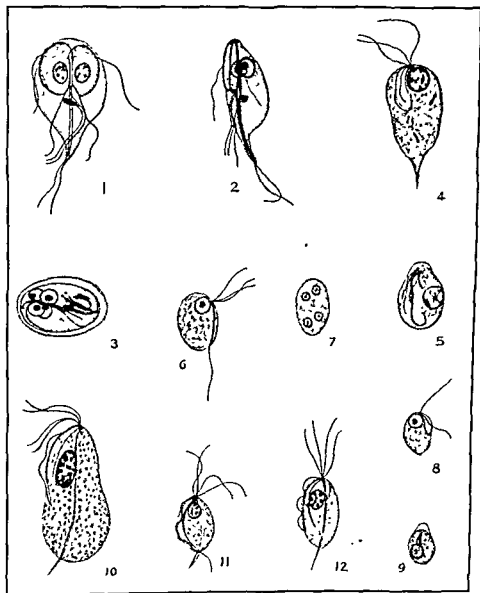


FIG. 66.—Flagellate protozoa of man ($\times 2000$). 1 and 2, Trophozoites of *Giardia lamblia*, dorsal and lateral views, 3, cyst of *G. lamblia*; 4 and 5, trophozoite and cyst of *Chilomastix mesnili*, 6 and 7, trophozoite and cyst of *Enteromonas hominis*, 8 and 9, trophozoite and cyst of *Retortamonas intestinalis*, 10, trophozoite of *Trichomonas vaginalis*; 11, trophozoite of *T. hominis*; 12, trophozoite of *T. tenax* (after D. H. Wenrich). (Heidenham's iron-alum hematoxylin stain) (Original)

differentiated from *T. vaginalis* by the presence of three to five anterior flagella and another flagellum which extends along the margin of the undulating membrane and trails from the posterior end of the body as a free flagellum. Otherwise, the nuclear and cytoplasmic structures are only slightly varied from those found in *T. vaginalis*. Physiological character-

istics, such as cultural requirements, are distinct from *T. vaginalis*. No cysts are known.

12. *Trichomonas tenax* (O. F. Müller, 1773). (Syn. *T. buccalis* Goodey, 1917 and *T. elongata* [Steinberg] Wenyon, 1926).—(a) *Trophozoite* (Fig. 66, No. 12).—Size, 6 to 12 μ . This organism inhabits the human mouth and although somewhat smaller, the morphological characteristics are similar to those of *T. vaginalis*. From available data, this species has a different incidence than *T. hominis* and *T. vaginalis* (Bland and Rakoff, 1937) and it also appears to be physiologically distinct from both the intestinal and vaginal species (Stabler and Feo, 1942). No cysts are known.

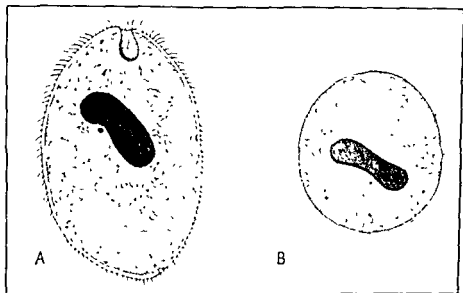


FIG. 67.—A, Trophozoite of *Balantidium coli*, B, cyst of *B. coli* ($\times 1000$) (Heidenhain's iron-alum hematoxylin stain) (Original)

13. *Retortamonas* (= *Embadomonas*) *intestinalis* (Wenyon and O'Connor, 1917).—(a) *Trophozoite* (Fig. 66, No. 8).—Size, 3.5 to 9 μ by 3 to 5 μ . This rare organism is spindle-shaped with one anterior flagellum and another thicker flagellum in the anterior cytostome.

(b) *Cyst* (Fig. 66, No. 9).—Size, 1 to 7 μ by 3 to 4 μ . The cysts are pear-shaped. Stained specimens show the nucleus and several structures which are the cytostome, fibrils and the flagella.

14. *Balantidium coli* (Malmsten) Stein, 1862.—(a) *Trophozoite* (Fig. 67, A).—Size, 30 to 200 μ by 20 to 100 μ . This protozoan inhabits the large intestine and is believed to be the only parasitic ciliate of man. The parasite is ovoid with a cytostome at the anterior end and with an anal opening or cytopyge at the posterior end. Cilia cover the entire body and they are used for locomotion and also for sweeping food particles into the cytostome. A small micronucleus is to be found usually in the concavity of the large kidney or bean-shaped macronucleus. Two contractile vacuoles can be observed in the posterior half of the cytoplasm.

(b) *Cysts* (Fig. 67, B).—Size, 45 to 65 μ in diameter. The cysts are usually spherical or nearly so and they are covered with a double transparent wall. Staining of the cysts reveals the nuclear components, and the cuticular markings with cilia can sometimes be differentiated.

This parasite is morphologically identical with a species of *Balantidium* commonly found in hogs, chimpanzees and the wild rat. In fact, current opinion backed by experimental evidence maintains that man obtains his infection from pigs.

DIAGNOSTIC METHODS

A laboratory examination for pathogenic bacteria and protozoa should be made on fecal samples from all patients with a diarrhea of unexplained

pathogens is strongly emphasized. Too many claims have been made for the pathogenicity of intestinal amœbæ and flagellates without ruling out bacteria as a probable cause for the symptoms attributed to protozoa.

The methods used for the diagnosis of intestinal protozoa are for the most part, applicable to the study of other human and animal protozoa. Several methods should be used on the same specimen to arrive at an accurate diagnosis. Although the use of all these methods is not indicated or practicable for every specimen, routine use of a staining method to accompany the direct microscopic examination is desirable for every sample.

I. **Collection of the Specimen.**—The specimen to be examined should be passed normally, if possible, and collected in a clean container, free of all soap, antiseptics or urine. Purged specimens give a higher percentage of positive results, but unless the examiner is skilled in identifying tissue cells and macrophages, as well as protozoa, these specimens are a source for error. Specimens passed after a dose of cascara sagrada are preferred by some workers for examination.

The specimens should be examined immediately or sent to the laboratory for prompt diagnosis. A fresh specimen is absolutely necessary to discover the relatively fragile active stages of the parasites. Cysts, when they are present, are more resistant to changes in environment and can be found in moist specimens for a variable period of time. Some organisms do not produce cysts, hence the urgent need for immediate examination. If the specimen must be stored for a short period of time before examination, storage in the ice box or even at room temperature is preferred to storage in the incubator at 37° C. because of the rapid overgrowth of bacteria at incubator temperature. The proper collection of the specimen is so important that supervision on the part of the responsible medical officer is often necessary to see that the correct procedure is followed.

II. **Methods for the Detection of Intestinal Protozoa.**—The following procedures are performed in making a careful and thorough fecal examination for protozoa. In following these directions, one must realize that protozoa inhabiting the intestinal tract are not always passed in the feces, so that a series of consecutive examinations, three to six, should be performed, if all protozoa are to be detected.

1. **Fresh Examination.**—A bit of feces from areas of the specimen containing blood and mucus or from the softer or liquid portion of the stool, is emulsified in a drop of physiological salt solution on the left side of a microscopic slide. Another bit is emulsified in a drop of Lugol's iodine solution (iodine crystals 5 gm., potassium iodide 10 gm., distilled water

to make 100 cc.) diluted to a port wine color on the right-hand side of the slide. The emulsions should not be too dense, but should permit one to see outlines of the hands of a watch or newspaper print through them. Cover glasses are added and a systematic search of the preparation is begun with a compound microscope. Search first with a 10x ocular and low power objective (10x) and then with the high dry objective (40x) to detect protozoa and their cysts or worm eggs and larva in heavy infections. The fresh preparation is the most useful in detecting and making a specific diagnosis of motile flagellates and *Balantidium coli*. The iodine preparation is most useful for the identification of amœbic trophozoites and cysts that were seen in the fresh preparation. The iodine not only immobilizes and kills the protozoa, but it stains the nuclei, differentiating them from the cytoplasm. The iodine preparation is invaluable too for detecting *Blastocystis hominis* and differentiating this troublesome plant-like organism from protozoa.

2. **Staining.**—Stained films should be made from all fecal samples to verify the diagnosis made in the fresh examination and to discover protozoa that might have been overlooked. The technic in general is the same for all intestinal protozoa, but some modifications are useful in order to obtain maximum results with the various species.

(a) **Fixatives.**—The most widely used and satisfactory fixative is Schaudinn's fluid (2 parts saturated aqueous mercuric chloride and 1 part 95 per cent ethyl alcohol) plus 5 per cent glacial acetic acid added at the time of using. This fixative may be heated to 45° C. for better results with amœbic cysts and ciliates. Time of fixation for thin films may be varied from five to fifteen minutes. One-half strength Schaudinn's fluid plus 2 per cent glacial acetic acid is very useful for small amœbæ and

fixative are very useful for fixation of tissues for the study of pathological lesions.

preparations but the procedure requires some technical skill for differentiation. The rapid technic of Johnson (1935) is given below because the procedure can be performed quickly and successfully for the diagnosis of amœbæ and flagellates.

method of Heidenhain gives the best definition of the protozoan nucleus in the staining of thin films and tissue sections. The associated pathology in lesions can be studied best after staining by routine pathological technics and also by the use of polychrome staining methods, such as eosin-methylene blue or Giemsa stains.

(c) *Technic.*—Johnson's⁹ rapid iron-alum hematoxylin technic:

- (1) Fix thin films on cover glasses or slides in hot (37° to 45° C.) Schaudinn's solution plus 5 to 10 per cent glacial acetic acid, ten minutes.
- (2) Ninety-five per cent alcohol plus iodine (port wine color), five minutes.
- (3) Seventy per cent alcohol, five minutes.
- (4) Rinse in tap water, one to three minutes.
- (5) Four per cent iron-alum solution (made from purple crystals), fifteen minutes.
- (6) Rinse in tap water, one to two minutes.
- (7) Stain in 0.5 per cent aqueous hematoxylin (10 cc. of a 5 per cent alcoholic [95 per cent ethyl alcohol] stock solution of hematoxylin plus 90 cc. distilled water), ten minutes.
- (8) Decolorize in 0.25 per cent iron-alum: amœbæ, twelve minutes; flagellates, six to ten minutes.
- (9) Wash in running water, three to thirty minutes.
- (10) Dehydrate in alcohol, clear in xylene. Mount.

The "long method" with Heidenhain's iron-alum hematoxylin is the preferred technic for making permanent mounts of intestinal protozoa and especially for staining *Balantidium coli*. This ciliate is difficult to differentiate in iron-alum solution. Although somewhat slower, differentiation with a saturated solution of picric acid in 70 per cent alcohol¹⁴ gives superior results.

Heidenhain's iron-alum hematoxylin technic:

- (1) Fix thin films in Schaudinn's solution plus 5 to 10 per cent glacial acetic acid, five to fifteen minutes.
- (2) Pass films through 50 per cent alcohol (five minutes) to 70 per cent alcohol. If the films are to be stained immediately, add iodine solution to give a straw color, leave for five minutes. This procedure extracts the crystals of fixative, otherwise do the same.
- (3) Alcohol 50, 30, 10 or for ten minutes.
- (4) Place in mordant solution (4 per cent iron-alum) and leave for two, twelve or twenty-four hours.
- (5) Rinse smears quickly in distilled water.
- (6) Place in hematoxylin solution (10 cc. of a 5 per cent alcoholic [95 per cent ethyl alcohol] stock solution of hematoxylin plus 90 cc. distilled water) for a time equivalent to that for the mordant solution.
- (7) Wash smears in distilled water for one minute.
- (8) Differentiate in a 2 per cent or 1 per cent solution of iron-alum, according to species of protozoa being stained. Control the staining by frequent examination of the film under the high or a water immersion objective. When the nuclear struc-
- (9) five minutes in each.
- (10) Clear the films in two changes of xylene.
- (11) Mount in gum dammar or clarite.

Mastery of the above methods provides preparations that are needed for the verification of diagnosis made in the fresh examination. The use of this staining procedure should be practised for each fecal sample.

3. **Cultivation.**—If the actual incidence of protozoa in the gastro-intestinal tract is desired, cultivation procedures are essential. The value of cultivation of feces for the detection of *Entamoeba histolytica* has been definitely established. This amoeba is a pathogen and any procedures to discover its presence are warranted in suspicious cases. *Balantidium coli* may be cultivated easily, also, but the size of this parasite enables easy diagnosis in the fresh examination. *Giardia lamblia* is the only intestinal flagellate of possible pathogenic importance which has not been cultivated, so that routine procedures to culture feces are essential only when attempting to detect *E. histolytica* or when making a survey for all intestinal protozoa. In fact, the low incidence of flagellates reported in most surveys is attributable to the failure to supplement the routine microscopic examination with culture technique. Flagellates, unless very numerous, are extremely difficult to discover in formed stools by ordinary fresh examinations and the study of stained films.

The most successful media in our hands for routine laboratory diagnosis and for surveys have been the *Entamoeba* medium (Difco) devised by Cleveland and Sanders, Boeck and Drbohlav's medium and Dobell and Laidlaw's medium. The first medium, with a modified liquid for overlaying the slants, is satisfactory for routine diagnosis, but the other two media are slightly better for maintaining cultures over prolonged periods of time. Although any of these media will permit growth of intestinal

and longer survival of *T. vaginalis*, but it has not been tried with intestinal flagellates.

(a) *Modified Cleveland and Sanders' Medium for the Cultivation of E. histolytica.*^{2,3,5}—This medium, consisting of liver infusion agar slants overlaid with serum-saline solution and with sterile rice starch added, is prepared as follows:

The liver infusion agar of Cleveland and Sanders can be bought as Bacto-Entamoeba medium (Difco). Dissolve 33 gm. in a liter of distilled water. Dispense a volume sufficient to make a slant of medium length with no butt in test tubes. Autoclave and slant, leaving the tubes at room temperature for several days to harden.

To make the fluid part of the medium, prepare a M/30 phosphate-salt solution, buffered at pH 8, by dissolving 11.23 gm. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.269 gm. of KH_2PO_4 and 8 gm. of NaCl in distilled water to make one liter. Autoclave at 15 pounds for twenty minutes, cool and add 10 parts buffer salt solution to 1 part sterile horse serum. Cover three-fourths of slant with this mixture and add 2 to 3 loopfuls of sterile rice starch to each tube. Incubate at 37° C. for twenty-four hours to test sterility. Final pH should be 7 to 7.2. Store tubes in refrigerator until needed for use.

To prepare sterile rice starch, place a few gm. of Difco Bacto-Rice Starch Powder in a culture tube (18 x 150 mm.). Place the tube horizontal in a hot-air oven at a temperature of 160° to 180° C. for one hour. Repeat

sterilization twice at twenty-four hour intervals. Care must be taken in this process to avoid chemical changes brought about by temperatures above those given.

(b) *Boeck and Drbohlav's*
break and emulsify in 75 cc
pense 4 cc. in culture tubes
in an inspissator and heat (70° C.) until the egg mixture has solidified. Now place the tubes in an autoclave at 15 pounds pressure for twenty minutes. Slants can be made also by placing tubes in the autoclave in a slanting position. Close the door and ports. Then turn on steam running the pressure quickly to 15 pounds and hold it there for ten minutes. Open the lower port and replace the air-steam mixture with live steam, maintaining a constant pressure of 15 pounds. When steam has replaced the mixture, close the lower port and maintain a pressure of 15 pounds for another fifteen minutes. Then cut off the steam and allow the autoclave to cool slowly. (Rigid precautions are taken to maintain constant pressure to prevent formation of air bubbles in slants.)

Cover the egg slants with 4 cc. of Ringer's solution—horse serum mixture, 10 parts to 1, and add 2 to 3 loopfuls of sterile rice starch to each tube. Incubate at 37° C. for twenty-four hours to test sterility. Store tubes in a refrigerator until needed for use.

(c) *Dobell and Laidlaw's Medium*.⁶—Undiluted sterile horse serum is used to make the slants. The serum is placed in tubes, slanted and inspissated at 80° C. for sixty to seventy minutes. Cool the tubes and cover the slants with the Ringer-serum solution used for Boeck and Drbohlav's medium. The buffer salt-serum mixture used for the modified Cleveland and Sanders' medium is also very satisfactory. Sterile rice starch is finally added and the tubes are incubated at 37° C. for twenty-four hours to test sterility.

(d) *Nutrient Agar Serum-saline Medium*.—Long slants without a butt are made in standard test tubes of nutrient agar (Difco, 1.5 per cent). These slants are covered one-half or three-fourths with a sterile Ringer's horse serum mixture in a ratio of 20 to 1. (Ringer's solution: NaCl 6 gm., KCl 0.1 gm., CaCl₂ 0.1 gm. NaHCO₃ 0.1 gm. distilled water 1000 cc.) The tubes with the lesser quantity are used for intestinal flagellates and with the greater quantity for *Trichomonas vaginalis*.

(e) *Trussell and Plaas Medium*¹³ for *Trichomonas vaginalis*.—Slants are made of liver infusion agar (Difco) and overlaid with a mixture (as for nutrient agar medium). Johnson¹⁰ obtained heavier growth by adjusting the agar and the solution to pH 5.8 with 1 N HCl and 0.25 per cent sodium phosphate. The addition of 0.2 per cent dextrose also enhances growth. Incubate tubes at 37° C. for twenty-four hours to test for sterility. Store the tubes in a refrigerator until needed for use.

To inoculate any of the above media, place fecal material or exudate about the size of a pea in the culture tube and mix thoroughly. Material from the more moist parts of the formed specimen or blood and mucus from a semi-formed or liquid specimen are most likely to contain protozoa. The tubes should be incubated for twenty-four hours at 37° C. and then examined.

freshly pla
eight hours

ing organisms. Amœbæ, if present, are most likely to be found on the surface of the sterile rice starch at the bottom of the tube. Flagellates are more actively motile and they swim about in the liquid portion of the culture. Careful and systematic search must be made of material withdrawn from the tubes because the organisms are seldom numerous in initial cultures.

If the media are correctly prepared and the technic of cultivation properly followed, :
Blastocystis
hominis in above media,
 and cause: or growth of
 protozoa. *E. histolytica*.

particularly in the tropics, where the majority of the population is infected with *Blastocystis*. Numerous attempts have been made to inhibit the growth or destroy *Blastocystis* during the process, but little success has been attained in this direction. In the presence of *Blastocystis*, cysts of *E. histolytica* can be hatched to initiate a culture by exposing a fecal suspension to 0.05 N HCl for thirty to forty-five minutes, neutralizing the reaction with 1 N NaOH, and inoculating sediment into the *Entamoeba* medium, incubating and examining it as above.

4. Concentration.—Within recent years Faust *et al.*⁷ have devised a successful concentration method for the detection of cysts of amœbæ and flagellates and eggs of various helminths. This method is of value only in the examination of semi-formed or formed stools and is of no value for trophozoites, because they are destroyed in the process. Carriers, who pass few cysts of *E. histolytica*, may be detected by the use of this method.

- (1) Thoroughly mix 1 part of a formed stool with 10 parts of lukewarm water in a glass container.
- (2) Strain 10 cc. of this mixture through one layer of wet cheesecloth, previously placed in a small funnel, into a Wassermann tube.
- (3) Place tube with filtrate in a centrifuge and spin for forty-five to sixty seconds (about 2500 revolutions per minute). Pour off supernatant, add 2 to 3 cc. of distilled water, shake thoroughly, and add water to fill tube. Spin in the centrifuge and repeat process as before until the supernatant is clear.
- (4) Pour off the clear supernatant fluid and add 3 to 4 cc. of a .53 per cent zinc sulfate solution (specific gravity 1.180). Mix sediment thoroughly in this solution and then add more of the same solution to within $\frac{1}{2}$ inch of the rim.
- (5) Spin in centrifuge for at least ninety seconds.
- (6) Remove several platinum loopfuls of material from the surface of the solution in the tube and place on a microscopic slide. Add a drop of dilute iodine solution and mix.
- (7) Add a cover glass and examine for cysts.

5. Serological Tests.—Since *E. histolytica* is the only common pathogenic protozoan in the intestine, this is the only protozoan infection which has been the subject of immunological study. Craig (1927) devised a practical complement-fixation test that has diagnostic value as a supplementary procedure. The test becomes positive within several days after the infection begins, but tapers off after the infection is cured, persisting up to one month. The reaction is specific, but difficulty in interpreting results, in cases of ulcerative colitis and in questionable infections with *E. coli*.

histolytica where no parasites can be demonstrated, detracts from its diagnostic value. The demonstration of *E. histolytica* is needed to support positive reactions in doubtful cases. On the other hand it is possible to find *E. histolytica* in the stool and have a negative complement-fixation reaction. Tissue invasion and the continued presence of antigen for the production of antibodies is necessary not only for the primary appearance of antibodies but for their persistence. Individuals losing or being cured of their infection become susceptible to a new infection.

(a) *Technic of the Complement-fixation Reaction.*—The technic of the complement-fixation test for amebiasis devised by Craig and modified by others, is essentially the same as the technic for the standard Wassermann reaction. Experience as a serologist is a prerequisite for the satisfactory performance of the test.

Although Craig used the antihuman hemolytic system, other workers have preferred the use of the antishcep hemolytic system. Reagents are prepared and titrated according to standard procedures for the system of choice. With the proper reagents and correct technic, success with the test is dependent upon the preparation of an antigen with high titre.

The antigen to be used is an alcoholic extract of cultures of *E. histolytica* containing trophozoites or cysts or of mucoid material from the intestines of a dog infected with *E. histolytica*. The cultures are not bacteria-free and hence they must contain a rich growth to provide a heavy suspension of amebae for extraction. The amebae are grown on the usual media in tubes or in flasks.⁸ At least 120 cultures are needed to produce sufficient organisms for extraction. The sediment containing the amebae of each culture is pipeted off, placed in suitable tubes and centrifugalized. The supernatant is discarded and $7\frac{1}{2}$ volumes of absolute alcohol are added. Extraction is carried out in the incubator at 37° C. for fifteen days, with daily shaking. Filter the mixture through a fine filter paper and titrate for potency.

An antigen prepared from cysts of *E. histolytica* washed free of bacteria is satisfactory also.¹² This antigen has the advantage of being pure but a comparison of results after using both antigens has failed to show marked difference.

The technic of performing the test is given below according to Craig⁴ (1942):

"Proceed as follows in making the tests: Place 0.9 cc. of normal saline (0.85 per cent) in all of the tubes used in the series of tests. In tube 1, anterior, place 0.1 cc. of the patient's serum that is being tested and the same amount in tube 1, posterior, and do the same in the case of every serum that is being tested. In tube 2, anterior and posterior, place 0.1 cc. of a known positive serum, and in tube 3, anterior and posterior, 0.1 cc. of a known negative serum. These are the controls. To each tube add 2 units of complement and to each anterior tube, 1 unit of the amebic antigen, and incubate all of the tubes in the water bath at 37° C. (98.6° F.) for one-half hour. At the expiration of this time add to each tube 0.1 cc. of the 5 per cent suspension of red blood corpuscles and 2 units of the amboceptor paper. The tubes are now incubated in the water bath at 37° C. for one hour, being thoroughly shaken every fifteen minutes during that period, in order to liberate the amboceptor from the paper. This is very important and if not done, false positive results will be obtained. At the end of the hour's incubation in the water bath the tubes are placed in the

ice-box for two hours and then the reactions are read, and, if the reagents have been properly titrated and used, the results should be as follows:

"The negative serum tube, tube 3, should show complete hemolysis; the positive serum tube, tube 2, should show complete inhibition of hemolysis; while tube 1, containing the serum to be tested, will if negative, show complete hemolysis, and if infection with *Endamaba histolytica* is present will show varying degrees of inhibition of hemolysis, usually either complete (4 plus) or almost complete, if treatment has not been administered. All of the tubes in the posterior row which do not contain antigen, should show complete hemolysis. If there is any inhibition of hemolysis in the posterior tubes something is either wrong with the reagents used in the test or the serum that is being tested is anticomplementary. If several different sera are being tested it will be noted that at the end of the hour's incubation in the water bath some will show complete hemolysis while others may show varying degrees of inhibition of hemolysis. If the tests were read at this time very erroneous results would be obtained but after two hours in the ice-box all of the tubes containing antigen should show complete hemolysis except those from individuals infected with *Endamaba histolytica*. The method of performing the complement-fixation test for amebiasis is graphically illustrated:

TABLE 83.—METHOD OF MAKING COMPLEMENT-FIXATION TEST FOR AMEBIASIS

Serum for Diagnosis	Positive Control Set	Negative Control Set
FRONT TUBES	FRONT TUBES	FRONT TUBES
Patient's serum, 0.1 cc.	Positive serum, 0.1 cc.	Normal serum, 0.1 cc.
Complement, 2 units	Complement, 2 units	Complement, 2 units
Antigen, 1 unit	Antigen, 1 unit	Antigen, 1 unit
Salt solution, 0.9 cc.	Salt solution, 0.9 cc.	Salt solution, 0.9 cc.
BACK TUBES	BACK TUBES	BACK TUBES
Patient's serum, 0.1 cc.	Positive serum, 0.1 cc.	Normal serum, 0.1 cc.
Complement, 2 units	Complement, 2 units	Complement, 2 units
Salt solution, 0.9 cc.	Salt solution, 0.9 cc.	Salt solution, 0.9 cc.
Incubate for one-half hour in water bath at 37° C.		
Add 2 units of amboceptor paper to each tube and 0.1 cc. of 5 per cent suspension of human red blood corpuscles.		
Incubate for one hour in water bath at 37° C. and let stand in ice-box for two hours, and then read reactions.		

(b) "Reading and Interpretation of the Reactions.—The reactions with (++) , plus (+) , plus-minus (*), and negative (—), according to the degree of inhibition of hemolysis, a 4 plus reaction signifying complete inhibition of hemolysis. In the interpretation of the reactions, any reaction below a strong 3 plus reaction is not considered as of diagnostic importance unless treatment has been administered or *Endamaba histolytica* is present in the stools. In the latter case, partial reactions may be of confirmatory value but a diagnosis should never be based upon such reactions alone."

III. Differential Diagnosis of Intestinal Protozoa.—The clinician requests a fecal examination primarily for the detection of *E. histolytica* and perhaps *Giardia lamblia* and *Balantidium coli*. In many cases of unexplained diarrhea, colitis, and dysentery, it is necessary to rule out infection by *E. histolytica* before appropriate treatment can be started. If no intestinal pathogens are found, the laboratory report will either be entirely negative

or will include only non-pathogenic protozoa. In the latter case the non-*Tricho-* of the so-called "flagellate diarrhea" but no experimental proof has ever been forthcoming. In such cases of diarrhea, tests for pathogenic bacteria as causal agents should be carried out. Although the crucial experiment to *irdia lamblia* must await the successful evidence for the pathogenicity of this

1. *Amœbæ*.—Diarrheic or dysenteric specimens usually contain only the active stages or trophozoites of amœbæ or flagellates. In cases where stools are passed very frequently, the organisms may be scarce because of dilution. a few trop their specific diagnosis is not so difficult. Mature cysts of *E. histolytica* contain four nuclei chromatoid bodies n larger than *E. histol*

Supernucleate cysts of *E. coli* containing 16, 32, or more nuclei are not uncommon. Uninucleate or binucleate cysts are more difficult to identify. If characteristic morphological features are not evident, examination of another specimen with identifiable cysts is preferable to an erroneous diagnosis. Cysts of *Iodamæba butschlii* may be confused with those of *E. histolytica*, but the irregular shape, a vacuole which stains deeply with iodine, and only one nucleus, will identify them.

The final and most specific differential character that distinguishes the

much larger karyosome is eccentric, and the peripheral chromatin is heavy and coarse. Other characters are given as diagnostic but they are too variable to be reliable. Differentiation of ectoplasm and endoplasm, presence of bacteria in the cytoplasm, motility and ingestion of red blood corpuscles depend on the nature of the stool specimen, and on the presence or absence of blood. Red blood corpuscles in trophozoite amœbæ are usually diagnostic for *E. histolytica* but there are even exceptions to this for trophozoites of *E. coli* can ingest red blood corpuscles (Fig. 64, No. 2) if they are present in the feces.¹⁵ Thus, nuclear structures are the most reliable characters for differentiating trophozoites of *E. histolytica* and *E. coli*. The only time that nuclei are difficult to distinguish is when the fecal specimen is old and the nuclei of the amœbæ are degenerate. In special cases where diagnosis is difficult to make, pathogenicity tests in kittens or young dogs may be used. This procedure is not practical for a routine laboratory test. Consecutive stool examinations to find diagnostic stages are preferable.

Differentiation of *Endolimax nana* and *Dientamæba fragilis* is not always easy in the fresh examination. The trophozoites are approximately the same size, so that the nuclear characters brought out by staining procedures will help to establish the diagnosis. Cysts of *E. nana* are distinctive, but no cysts are known for *D. fragilis*. The latter amœba has been suspected of having pathogenic properties since the organism is most commonly

found in diarrheic stools. In all these cases, however, pathogenic bacteria were not ruled out as causal agents of the condition. Thus, until more experiments are forthcoming, the pathogenicity of this parasite cannot be established.

2. **Flagellates.**—Since *Giardia lamblia* has not been cultivated, crucial experiments to prove its pathogenicity cannot be performed. This parasite is the only species which lives in the small intestine, and the incidence of infection ranges up to 50 per cent in children down to approximately 10 per cent in adults. Infection is frequently associated with a mucoid diarrhea, vague gastro-intestinal symptoms, loss of weight, and anemia. Cure of the infection with the specific drug, atabrine, causes the symptoms to disappear in the majority of cases.

G. lamblia is most frequently found in the cystic stage in stool specimens. Trophozoites generally occur only in semi-liquid or liquid feces because of their habitat in the small intestine. Both stages are distinctive in their morphology and diagnosis is not difficult (Fig. 66, Nos. 1 and 2). The trophozoites may occur in great numbers enmeshed in the mucoid plaques which are commonly found in a positive specimen. This infection does not always produce symptoms, and such "symptomless carriers" are of medical interest only as a source of infection for others.

Trichomonas hominis (Fig. 66, No. 11) has an undulating membrane which distinguishes it from other intestinal flagellates. Jerky, rotating movements are also characteristic. No cysts are known. Great numbers may be detected in liquid stools but they are not pathogenic. The liquid stool merely provides a favorable environment in which the flagellates multiply rapidly. This species is distinct morphologically and physiologically from *T. tenax* (= *T. elongata*) found in the human mouth and *T. vaginalis* which is the cause of trichomonas vaginitis in women. *T.*

cultural and staining properties of *T. vaginalis* are also distinct from *T. hominis*. In cultures, the morphological characters and physiology of each species remain distinct.

The other three species of flagellates, *Chilomastix mesnili*, *Enteromonas hominis*, and *Retortamonas intestinalis* (Fig. 66, Nos. 4 to 9) exist as trophozoites. Trophozoites of *Chilomastix mesnili* move slowly. An oral groove with a flagellum is characteristic. The cysts are pear-shaped.

R. intestinalis is a very small flagellate, with an oral groove containing a flagellum and another flagellum extending anteriorly. The cysts are pear-shaped also but smaller than those of *Chilomastix*. *E. hominis* is most commonly found in diarrheic stools or with the aid of cultures. The morphology of the trophozoites and cysts is characteristic (Fig. 66, Nos. 6 and 7).

3. **Ciliates.**—*Balantidium coli* is the only parasitic ciliate of man. This species causes balantidiasis. Trophozoites may occur singly or in pairs. They are large, pear-shaped, and ciliated with two long cilia which are used for locomotion. The anterior cilia are used for food getting. The posterior cilia are used for locomotion.

plasm along with the sausage-shaped macronucleus and spherical micronucleus. The resistant cysts or infective stages are spherical with a diameter of 45 to 65 μ . In stained preparations, the nuclei can be differentiated and the striations and cilia of the cell wall can be seen. Cultivation of this organism is successful by a variety of methods.

4. Sporozoa.—*Isospora hominis* is a rare parasite of man,¹¹ but it has been described from various countries of the world. Only the spores have been studied (Fig. 68); the tissue stages are still unknown. This organism is suspected of pathogenicity, but until more information is available, no definite statements can be made.



FIG. 68.—*Isospora hominis* Fantham, 1917 ($\times 1000$). a, Oöcyst in stool at time of passage, b, beginning formation of two sporocysts, c, sporocysts formed (thirty-six hours), large residual mass, sporozoites not completely formed; oöcyst wall ruptured by pressure; d, mature oöcyst (fifty-six hours). (Magath, courtesy of the Amer. Jour. Trop. Med.)

IV. Additional Aids in Fecal Diagnosis.—Reliable fecal diagnoses are only made after considerable study of known species of parasites and actual experience with fecal specimens. The typical textbook figures of diagnostic stages are not always present in samples. Furthermore, the infinite variety of objects ordinarily found in feces leads to a great amount of confusion on the part of an inexperienced examiner.

1. Sources of Error in Diagnosis.—Vegetable fibers, undigested cellulose particles of plant foods, yeast cells, undigested muscle fibers, and crystals of fatty acids and soaps may be present in the feces. Tissue cells derived

from the intestine and exudative cells, such as leucocytes and macrophages, occur frequently. In fresh normally passed or purged specimens the leucocytes and macrophages may be seen along with debris and red blood cells.

Cysts of coprozoic protozoa may be mistaken for those of parasitic intestinal organisms. The yeast-like *Blastocystis hominis* may assume a great variety of sizes and shapes, resembling cystic stages of amœbæ or flagellates (Fig. 65, Nos. 10 and 11). This organism is particularly pleomorphic, but if careful observation is made in comparing the structure of amœbic cysts with blastocysts, differences may be recognized easily. The use of iodine is recommended in staining *Blastocystis*, because of the way iodine causes the material in the central vacuole to shrink away from the outer layer of cytoplasm.

Charcot-Leyden crystals (Fig. 65, No. 12) are frequently found in dysenteric specimens. The frequency of their occurrence in amœbic dysentery is believed by many to warrant suspicions of infection with *E. histolytica* in case no stages of the parasite are found. No diagnosis of amœbic dysentery should be made without finding the amœbæ, because Charcot-Leyden crystals may occur in ulcerative colitis, severe diarrhea and other infections of the intestine.

TABLE 84.—DIFFERENTIAL DIAGNOSIS OF BACILLARY AND AMŒBIC DYSENTERY (CALLENDER*)

Exudate	Bacillary dysentery	Amœbic dysentery
Blood	Varying amounts	Small amounts to actual hemorrhage
Polymorphonuclear leukocytes	About 90 per cent exudate. Many show nuclear degeneration (ringing). Cytoplasm frequently contains fat.	Few. Cytoplasm of some of these present shows degenerative changes and in such the nuclei may appear pyknotic.
Endothelial macrophages	Present in varying numbers. Actively phagocytic frequently contain erythrocytes and leucocytes. Undergo toxic degeneration; "ghost cells."	Not seen except in cases also having bacterial dysentery.
Plasma cells	Present, relatively more abundant early.	Present in small numbers.
Pyknotic bodies	Proportionately insignificant, but are found.	Constitute about 80 per cent of cellular elements.
<i>E. histolytica</i> trophozoites	Absent unless the two diseases are both present.	Present and must be found to make diagnosis.
Amount of exudate, actual hemorrhage excluded	Massive, a large part of the stool.	Small.
Bacterial content	Low.	Very high, usually.

* CALLENDER, G. R.: The Cytological Diagnosis of Dysenteric Conditions and Its Application in the Military Service, Mil. Surg., 56, 650, 1925.

2. **Cellular Pathology of Dysenteric Specimens.**—After examining a number of dysenteric specimens, one is impressed by the great differences in their cellular content. Study of tissue sections from amœbic, balantidial, and bacillary dysentery, and of ulcerative colitis will reveal the fundamental basis for the characteristic histopathology and cellular exudate which is passed in each infection. Proctoscopic examinations also reveal striking differences between the highly diffuse inflammation of the mucosa in bacillary dysentery and the ulceration without extensive inflammation in amœbic dysentery. Cases of amœbic dysentery may become secondarily involved with bacterial infection to confuse the picture, but the nature of the exudate as carefully worked out by Callender (Table S4) is of distinct diagnostic value.

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CHAPTER XXXVI

BLOOD PROTOZOA

By QUENTIN M. GEIMAN

MALARIA PLASMODIA

THE great importance of malaria as a disease in time of peace is far overshadowed by its importance in wartime. In World War II, great numbers of men are travelling to and from highly endemic areas. Armed forces are stationed or are fighting in many tropical and subtropical regions where malaria is hyperendemic (Fig. 70). Troops may be invalided home, bringing with them parasitic infections acquired in tropical areas. Troops returning from malarious areas after the war is over will be dispersed to their homes throughout this country. If such individuals are infected with malaria and the local anopheline mosquito vectors gain access to them, local epidemics of malaria can be expected.

Problems arising from the presence of large numbers of troops in malarious areas place a distinct obligation on each laboratory diagnostician in the armed forces or the civilian health services who is responsible for the diagnosis of malaria parasites. cursory examination of textbooks or stained films containing parasites provides insufficient background for arriving at a reliable and accurate diagnosis. Some concentrated training from skilled and experienced experts is needed in order to satisfy the necessary requirements.

The following consideration of malaria is devoted to a description of the parasites and methods for diagnosing infection, but insufficient space is available to present a complete account of this small but important part of malariology. If additional information is desired, recent textbooks of tropical medicine and monographs on malaria should be consulted.

I. Classification.—The parasites of human malaria belong to the class *Sporozoa*, order *Hæmosporidia* and genus *Plasmodium*. Although other mammals, birds, and certain lower animals harbor malaria parasites, only four species occur in man.

Species of human malaria parasites:

Plasmodium falciparum of malignant tertian (æstivo-autumnal or subtertian) malaria with a forty-eight hour cycle.

P. malariae of quartan malaria with a seventy-two hour cycle.

P. vivax of benign tertian malaria with a forty-eight hour cycle

P. ovale of ovale tertian malaria with a forty-eight hour cycle

II. Life Cycle.—The complete life cycle of the plasmodia of man includes both asexual and sexual stages of development or "alternation of generations" between the intermediate and definitive host. Although the immature sexual stages or gametocytes develop in the peripheral blood of man coincident with the asexual cycle, the process is completed only within the female anopheline mosquito which ingests blood containing the sexual stages. After the required developmental period, which varies with the temperature of the environment, infective stages are produced in the

mosquito which lead to the transmission of the disease when the mosquito ingests another human blood meal.

1. **Cycle in Man.**—When an infective anopheline mosquito bites man and inoculates sporozoites (the infective stage of the parasite) (Fig. 69, No. 7), a chain of events is initiated which leads to the development of clinical malaria. During the subsequent incubation period, which may vary from eleven to fourteen days to several months, the site, nature and fate of the process which results in the blood stream infection is entirely obscure. Experiments have shown that the peripheral blood does not contain detectable parasites or become infectious for a number of days after the infective bite. Thus, the sporozoites apparently undergo some necessary development in the tissues before the required stage is produced for the penetration of the red blood corpuscles.

In a high percentage of cases, the parasites can be detected in the blood before the beginning of clinical signs and symptoms. The youngest form to be found in the erythrocytes is the "signet ring" with a central vacuole and a ring of cytoplasm containing a chromatin dot or granule (Plate IV, Fig. 2). The growth of the parasite proceeds gradually at the expense of the host cell and the length of time required to complete the asexual process depends upon the species involved. The vacuole disappears and the volume of the parasite increases as it grows. The stage containing only one chromatin mass is called a trophozoite. As growth continues, pigment granules are produced and the chromatin mass divides to form the stage known as a schizont (Fig. 16). Several divisions of the chromatin in the schizont take place to form the fully grown parasite or the segmenter which may almost completely fill the cell (Fig. 20). At the time of the paroxysm, the red cell ruptures to free pigment and the merozoites, which were formed by segmentation, into the plasma of the blood. The merozoites immediately invade new red blood cells to initiate a new cycle or are ingested by phagocytes. The same asexual process can be repeated to form a new brood of merozoites or the parasites can develop into male and female gametocytes (Fig. 23 and 24), which completely fill the erythrocytes and contain one chromatin mass only.

2. **Cycle in the Mosquito.**—When a patient with clinical malaria or a carrier of malaria produces a certain concentration of gametocytes, the blood will lead to infection in an anopheline mosquito when the blood is ingested. In the blood-filled stomach of the mosquito, the male gametocyte or microgametocyte undergoes a process of "exflagellation" or throwing off of four to eight slender microgametes (Fig. 69, No. 2). The macrogametocyte undergoes a change too (Fig. 69, No. 1) after which a microgamete penetrates it and fuses with the nucleus to form a fertilized cell or zygote. Elongation takes place and the cell, now called an ookinete (Fig. 69, No. 3) becomes motile. The ookinete penetrates the lining of the mosquito's stomach, migrates through between the epithelial cells to lie under the outer membrane of the stomach (Fig. 69, No. 4) and begin the formation of the oöcyst. The oöcyst begins to grow (Fig. 69, No. 5) and after numerous nuclear divisions, sporozoites are differentiated. The oöcyst eventually ruptures (Fig. 69, No. 6), freeing the sporozoites into the body cavity of the mosquito, leading to their penetration of the tissues and fluids. The sporozoites which penetrate the salivary glands (Fig. 69, No. 7) are the most important because their injection along with saliva



FIG. 69.—Stages in the sexual development of the malaria parasite. ($\times 1100$ except No. 5.)

1, Macrogametocyte of *P. vivax*; 2, "exflagellation" or formation of microgametes from microgametocyte, *P. vivax*; 3, ookinete, *P. vivax*, from stomach of mosquito; 4, ookinete, *P. relatum*, in stomach wall of mosquito, *Culex pipiens*; 5, oocysts, *P. vivax*, on stomach of mosquito ($\times 25$); 6, oocyst breaking into body cavity to free sporozoites; 7, sporozoites penetrating the salivary gland. (Photographs 4 and 6, made from slides in the collection of Dr. L. R. Cleveland.)

at the next feeding leads to the transmission of the disease. This whole process or sporogenous cycle in the mosquito requires a definite time, depending upon the temperature of the environment. At 20° to 21° C., the salivary glands of an anopheline mosquito infected with *P. falciparum* become positive for sporozoites in about twenty-two to twenty-three days, with *P. vivax* in about sixteen to seventeen days, and with *P. malariae* in thirty to thirty-five days.

Description and Differential Diagnosis of Human Plasmodia

Benign tertian and malignant tertian malaria are the types of this disease most frequently encountered. Quartan malaria is of importance in certain parts of Africa and India, but has a tendency to be localized in its distribution wherever it occurs. Ovale tertian malaria is of little importance as a disease entity from a public health viewpoint.

Although generalization is hazardous when dealing with malaria, infections with the above four species of parasites differ in their symptomatology, latency, and response to anti-malarial drugs. *Plasmodium falciparum*, causing malignant tertian malaria, is highly pathogenic and a common tropical infection. The great invasiveness and pathogenicity of *P. falciparum* makes the specific diagnosis of this parasite extremely valuable to the clinician. Obviously, a patient infected with *P. falciparum* should be watched more carefully and treated differently than the patient with *P. vivax*. Thus specific diagnosis becomes of great importance to the medical officers of the armed forces operating in the endemic and hyperendemic malarious areas in the tropics, subtropics and temperate zones.

I. Description of Parasites.—1. *Plasmodium Falciparum*.—Infection of the peripheral blood with this species is usually characterized by the presence of "rings" only or "rings" and gametocytes. The trophozoites

LEGEND FOR PLATE III.—*P. falciparum*

1. Very young ring form trophozoite.
2. Double infection of single cell with young trophozoites, one a "marginal form," the other "signet ring" form.
- 3, 4. Young trophozoites showing double chromatin dots.
- 5, 6, 7. Developing trophozoite forms.
8. Three medium trophozoites in one cell.
9. Trophozoite showing pigment, in a cell containing Maurer's spots.
- 10, 11. Two trophozoites in each of two cells, showing variation of forms which parasites may assume.
12. Almost mature trophozoite showing haze of pigment throughout cytoplasm. Maurer's spots in the cell.
13. Aestivo-autumnal "slender forms."
14. Mature trophozoite, showing clumped pigment.
15. Parasite in the process of initial chromatin division.
- 16, 17, 18, 19. Various phases of the development of the schizont ("presegmenting schizonts").
20. Mature schizont.
21. Mature gametocyte.
22. Immature microgametocyte.
23. Mature microgametocyte.

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PLATE III



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MALARIA

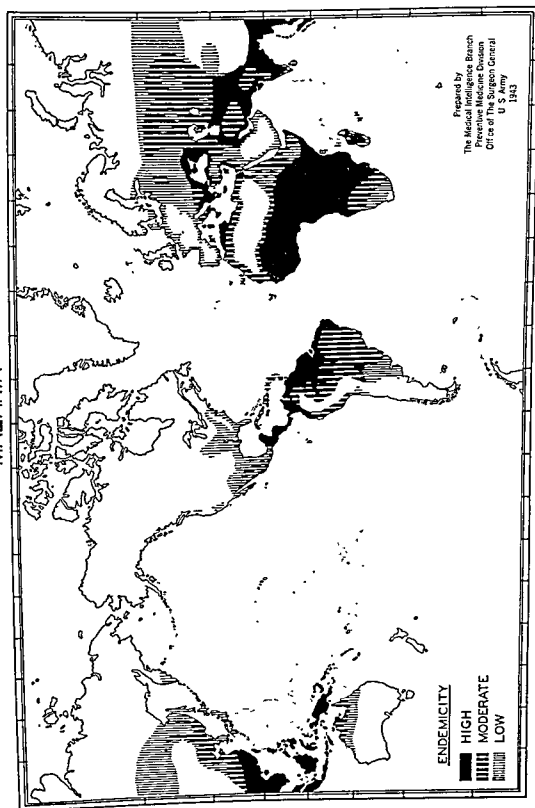


FIG 70 —Geographical distribution of malaria.

and schizonts develop within the small capillaries of the viscera and other internal organs. The presence of developing asexual stages in the peripheral blood is usually a bad prognostic sign because it signifies blockade and stasis of blood in the small capillaries. This phenomenon causes serious involvement of the brain, intestines, kidneys and other complications in this infection.

Two or more "rings" of *P. falciparum* may parasitize a single red cell. (Plate III, Fig. 8.) The "rings" have a delicate appearance and there may be two chromatin granules (Fig. 3). Multiple infection of the red cell is usually diagnostic for this species. Red and irregularly shaped dots, known as Maurer's "spots" (Fig. 9) may stain in the cytoplasm of the parasitized red cell.

When the older asexual stages are found in the peripheral blood (Figs. 14-19) or are studied in impression films of organs, the parasite seldom attains the size of *P. vivax* or *P. malariae*. The schizont and segmenter fill only about two-thirds the diameter of the erythrocyte. The merozoites number 8 to 24 and the pigment is black or brownish-black in color (Fig. 20).

The gametocytes, often called "crescents," of this species have a distinct sausage shape. The parasite elongates to form rounded ends (Figs. 23-25). The tightly stretched membrane of the red cell may be seen occasionally in the concavity of a mature gametocyte (Fig. 26). During the growth process, the single mass of chromatin and pigment become aligned in the center of the body. Slight differences in size, shape and stainability aid in distinguishing the two types of gametocytes (Figs. 26 and 28). These sexual stages are not always found in the peripheral blood. In a primary attack they seldom are found before the tenth day after the initial paroxysm. In terms of total parasite count, which may reach 300 to 500 thousand per c.mm. in acute cases, they may be non-existent or occur in great numbers.

LEGEND FOR PLATE IV.—*P. vivax*

- 1 Normal sized red cell with marginal ring form trophozoite.
- 2 Young signet ring form trophozoite in a macrocyte
- 3 Slightly older ring form trophozoite in red cell showing basophilic stippling
- 4 Polychromatophilic red cell containing young tertian parasite with pseudopodia.
- 5 Ring form trophozoite showing pigment in cytoplasm, in an enlarged cell containing Schüffner's stippling *
- 6, 7. Very tenuous medium trophozoite forms.
- 8 Three ameboid trophozoites with fused cytoplasm.
- 9, 11, 12, 13 Older ameboid trophozoites in process of development.
10. Two ameboid trophozoites in one cell.
- 14 Mature trophozoite
- 15 Mature trophozoite with chromatin apparently in process of division.
- 16, 17, 18, 19. Schizonts showing progressive steps in division ("presegmenting schizonts").
- 20 Mature schizont.
- 21, 22. Developing gametocytes.
23. Mature microgametocyte
- 24 Mature macrogametocyte.

* Schüffner's stippling does not appear in all cells containing the growing and older forms of *P. vivax* as would be indicated by these pictures, but it can be found with any stage from the fairly young ring form onward.

PLATE IV



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2. *Plasmodium Vivax*.—This species is a common parasite, causing benign tertian malaria. All stages of the asexual cycle develop in the peripheral blood, requiring forty-eight hours for completion of the cycle. During the initial paroxysms, young and fully grown stages may occur simultaneously in the blood stream, but then the cycle synchronizes or "gets in step" with the majority of the parasites maturing at the same time; a fact which is the basis for the typical fever cycle of tertian malaria.

The "rings" of this species (Plate IV, Figs. 1-3) are slightly heavier than those of *P. falciparum* and only rarely does one find a cell containing two "rings."

tion of fine do

plasm (Figs. 5

which persist in the parasitized cell, are diagnostic of the species.

ocyte, and loses its vacuole

Yellowish-brown pigment as the parasite becomes older. Division of the chromatin mass begins after the parasite practically fills the cell. Twelve to twenty-four masses (average 16) of chromatin are produced in the schizont to form the segmenter (Figs. 16-20). When segmentation is complete, the cell membrane ruptures to release the merozoites and the massed pigment granules into the plasma of the blood. The cycle then is repeated as the merozoites parasitize more normal red blood cells. The density of infection seldom exceeds 100,000 per c.mm. of blood.

The sexual stages or gametocytes of *P. vivax* may be found in the blood during the first or second paroxysm. These stages are spherical, almost filling the red cell and containing only one chromatin mass and pigment granules, which are diffusely scattered throughout the cytoplasm of the parasite. Although it is difficult to distinguish fully grown trophozoites from immature gametocytes, the mature micro- and macrogametocytes have characteristics which differentiate them (Figs. 23-24).

3. *Plasmodium Malariae*.—This species causes quartan malaria, with a typical asexual cycle of seventy-two hours. The "ring" forms (Plate V, Figs. 1 to 5) are very similar to those of *P. vivax*, but this parasite does not cause enlargement of the erythrocytes or the formation of "Schuffner's granules." The growing trophozoites frequently assume an equatorial or band-like position in the red cell (Figs. 6, 10, 13). This characteristic is diagnostic and persists during the growth of the trophozoite. Young *et al.* have shown that the trophozoite stage exists for 54.2 hours, young schizont for 10.4 hours, and the segmenter for 7.4 hours.

P. malariae produces a smaller number of merozoites, 6 to 12, which are usually arranged in a "rosette" form around the central mass of pigment (Fig. 20). The pigment granules of this species are very coarse and dark brown in color.

Since the density of parasites in quartan malaria seldom exceeds 20,000 per c.mm., there is a basis for the relatively scant numbers of gametocytes produced. The gametocytes are spherical in shape almost filling the red blood cell (Figs. 21 to 24). Except for the coarser pigment and smaller diameter, their characteristics are similar to those of *P. vivax*.

4. *Plasmodium Ovale*.—The evidence now available has established the specificity of this parasite. This organism causes a mild infection with a

typical forty-eight hour cycle. The characteristics of the parasite (Fig. 71) are somewhat similar to those of *P. malariae*, but the effect in the red cell resembles more closely the appearance produced by *P. vivax*. The red cell enlarges to assume a specific ovoid shape with an irregular fringed margin. "Schüffner's granules" are present in almost 100 per cent of the infected erythrocytes. Although the trophozoites and schizonts do not assume a band-like position and the pigment is not as coarse, the segmenters resemble those of *P. malariae* and produce approximately the same number (8) of merozoites.



FIG. 71 — Asexual stages of *Plasmodium ovale*. The characteristic oval shape and dense stippling of the infected red cell can be seen. $\times 1100$.

The gametocytes cannot be distinguished from those of *P. vivax*. The presence of "Schüffner's granules" and the enlarged erythrocyte differentiates them from *P. malariae*. Mosquitoes have been infected with *P. ovale* and experimental transmission of the disease has been achieved. Throughout the experiments, the parasite has maintained its characteristics and the disease has been consistent in its clinical features.

LEGEND FOR PLATE V.—*P. malariae*

1. Young ring form trophozoite of quartan malaria
- 2, 3, 4 Young trophozoite forms of the parasite showing gradual increase of chromatin and cytoplasm.
5. Developing ring form trophozoite showing pigment granule.
- 6 Early band form trophozoite—elongated chromatin, some pigment apparent.
- 7, 8, 9, 10, 11, 12. Some forms which the developing trophozoite of quartan may take.
- 13, 14 Mature trophozoites—one a band form.
- 15, 16, 17, 18, 19 Phases in the development of the schizont ("presegmenting schizonts").
- 20 Mature schizont.
21. Immature microgametocyte
22. Immature macrogametocyte.
- 23 Mature microgametocyte.
24. Mature macrogametocyte

PLATE V



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II. Differential Diagnosis.—In attempting to arrive at a specific diagnosis of malaria parasites, an important point to remember is that the parasites go through a constantly changing periodic cycle of development in the blood or internal organs of the host. The process is not static and if a decision cannot be made from a single blood sample, additional films should be made at intervals and particularly at a time of the cycle when the diagnostic stages can be obtained. For example, the characteristic trophozoites of *P. vivax* are obtainable up to twenty-four hours after the paroxysm. The band-forms of *P. malarix* from twenty-four to sixty hours after the paroxysms and the "segmenters" approximately sixty-five hours after the paroxysm. In cases of chronic malaria, persistent search of consecutive blood films is frequently necessary before the parasites are found. Unsuspected cases of latent malaria are occasionally discovered when an infected individual gives blood for a transfusion.

Double infections with one species may also be present in the same patient. If the parasite involved is *P. vivax*, a cycle of the development may be completed each day to give a "double tertian" or quotidian fever. Mixed infections of two species or even three may be contracted by the same patient in countries where two or more species are prevalent. Consecutive attacks with different species of plasmodia are also common in highly malarious countries, but there is evidence to show that these patients harbor mixed infections and only show attacks of a single species because of species antagonism, first allowing one and then the other to dominate.

Under the above description of each parasite, the various stages which are diagnostic were indicated. Frequently in routine diagnosis, the typical textbook diagnostic stages are not found. Nevertheless, if the examiner is thoroughly familiar with the morphology and development of each parasite in the human host, the stage of the parasite and the species can usually be determined without any difficulty.

For *P. vivax*, the enlarged red cell, "Schüffner's granules," ameboid appearance of trophozoite, characteristic appearance of the segmenter and numbers of merozoites are diagnostic.

For *P. malarix*, no enlarged red cell, no stippling, the equatorial or band-form of trophozoite, coarse brown pigment, and the characteristic appearance of the segmenter in a "rosette" form with the production of 6 to 12 (average 8) merozoites are diagnostic.

For *P. falciparum*, no enlarged red cell, Maurer's dots (may not be present), multiple infection of red cell with "rings," absence of older asexual forms in the peripheral blood, and the sausage-shaped or "crescent"

If a specific diagnosis is difficult to make in the center of the film, parasites at the margin usually maintain their form and can be identified more easily. When only "ring" forms are present, it is particularly necessary to find and identify the multiple cell infection of *P. falciparum* at the margin. "Crescents" of *P. falciparum* (Plate VI) are easily identified when present. The "Schüffner's granules" and shape of the trophozoites and schizonts of

P. vivax (Plate VII) help to identify tertian malaria. The stages of *P. malariae* are usually the smallest with coarse pigment, and with either the band-like form of the trophozoite or "rosette" segmenter (Plate VIII) present to aid in the diagnosis. For more complete descriptions, the papers of Field and Le Fleming¹ and of Wilcox² contain detailed accounts.

III. **Puzzles and Sources of Error in Diagnosis.**—Clinicians and medical officers frequently request confirmation of a diagnosis of malaria. All too frequently, negative films are reported as positive and errors on the part of the original examiner are traceable not only to lack of knowledge about the parasites, but to errors in technic or to the presence of abnormal blood cells, bacteria, fungi, and extraneous debris that was picked up during the staining process and storage of films.

Blood platelets superimposed on red blood cells may be mistaken for malaria parasites. Abnormal and enlarged platelets are diagnosed as "crescents" of *P. falciparum*, even though their size is much smaller. Free-living protozoa, growing in the buffered water, or contaminating staining jars cause difficulty in the tropics. Films prepared in the field under a variety of atmospheric conditions may contain bacteria, yeasts and fungi which settle on them from dust-laden air during the drying process. In areas where leishmaniasis occurs, Leishman-Donovan bodies which are free in the blood plasma, superimposed on red blood cells, or present in fragments of macrophages, may lead to confusion. In other words, a variety of objects may simulate the appearance of malaria parasites in stained blood films. No diagnosis of malaria should be made unless the parasite can be definitely identified. If there is any question of identity, additional films should be obtained and examined.

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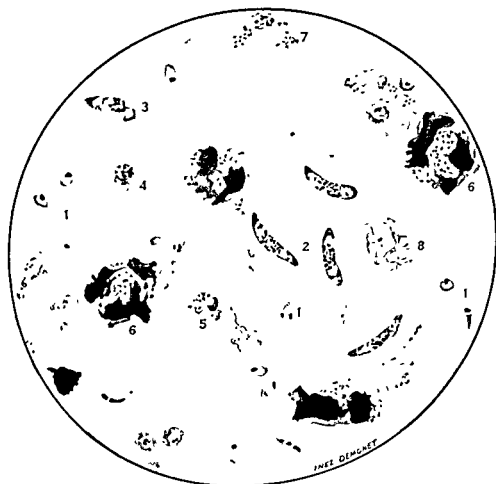
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Laboratory Diagnosis of Malaria

Malaria, a disease produced by four distinct species of parasites, causes a great variety of clinical symptoms and atypical states of infection. In latent malaria, no symptoms may occur for a long period of time. Clinical, pathological and physiological studies are not of specific value for diagnostic purposes. Consequently, a diagnosis of malaria is entirely dependent at the present time on finding and identifying the parasites in the blood.

The current laboratory methods of preparing thin and thick films and the satisfactory staining of these films have become simplified enough to be mastered by anyone with facilities for using the microscope. Routine methods for the preparation of thin films for blood studies may be followed but a special thick film technic is now practised by malariologists for routine diagnosis and conduct of surveys. The latter method is advantageous for the discovery of a low concentration of parasites and for saving time in conducting the examination. The thick film technic is also extremely valuable in the diagnosis of relapsing fever and trypanosomiasis.

PLATE VI



P. falciparum—thick film

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I. Preparation of Films.—Thin films should be prepared on microscopic slides or cover glasses that are scrupulously cleansed of dirt, dust and grease. In making the film on a slide, the slide for spreading the blood should be brought in gentle contact with the drop at a 30 degree angle and then with an even motion the film is spread. Under no circumstances should the slide for spreading the film be pushed through and across the drop of blood to crush the red blood cells. The thin film should dry quickly and show a single layer of well separated erythrocytes.

The thick film, a concentration method, is prepared by placing a large drop of blood on a thoroughly clean slide and then with the corner of another slide, a needle or other instrument and a rotating motion, the drop is spread so that the erythrocytes are several layers thick in the center tapering off to a single layer at the margin. One should be able to read print or see the hands of a watch through the thickest part of the film. The film should dry flat and be protected from dust and flies during the process. If the film is to be stained as quickly as possible, or if films are being made in the humidity of the tropics, place in an incubator at 37° C. for one hour, otherwise at room temperature overnight. The film should not be heated to a higher temperature to hasten the process because the cells will be fixed and dehemoglobinization of the red blood cells will be prevented during the staining process. The films should be stained preferably within forty-eight hours because of degenerative changes which take place in the blood drop.

Thin and thick films may be placed on the same slide to facilitate the verification in the thin film of parasites found in the thick film. This procedure is also economical in conducting large surveys. All slides should be carefully labelled with a diamond or wax pencil, or a sharp lead pencil may be used to write the legend on the thin film.

II. Staining.—Any polychrome or Romanowsky stain such as Wright's, Leishman's, Hastings' or Giemsa may be used to stain malaria parasites. Of these stains, Giemsa is used most widely, because of the greater precision and differentiation obtained between structures of the parasites and the host cells. After the choice of stain is made, the required technic should be mastered so that no infections are missed because of faulty technic. Wright's staining technic, in particular, should work well before being used for the diagnosis of malaria parasites. Prior to the present war, Grubler's Giemsa stain of German origin was highly satisfactory and easily obtained. As a result of the deserving efforts of Roe, Lillie, and Wilcox⁴⁴ certified American-made Giemsa stain is now available (National Aniline and Chemical Co., Inc., N. Y.) for the procedure. This stain may be used for thin and thick films or combined with Wright's stain for a rapid technic in staining thick films.

Giemsa Stock Staining Solution.—Dissolve 0.5 gm. of powder (National Aniline and Chemical Co., Inc., N. Y.) in 33 cc. of glycerol (pure reagent) at 55° to 60° C. for one and a half to two hours. To this add 33 cc. of absolute methyl alcohol (acetone free). Mix thoroughly and allow to sediment overnight in a desiccator to prevent absorption of moisture. Pour off into small bottles (30-cc.) and stopper tightly. To use, various dilutions of stain, up to 3 per cent with buffered water may be made to obtain the desired results.

Wright-Giemsa Stain.²—Dissolve 2 gm. Giemsa powder (National Aniline Chemical Co., Inc., N. Y.) in 100 cc. glycerol (C. P. from a freshly opened bottle) with the aid of heating in a water bath at 55° to 60° C. for two hours. Precautions must be taken to avoid absorption of moisture during the process and when the mixture is stirred at intervals. To this mixture add 100 cc. of Wright's staining solution (aged solution of 2 gm. powder [National Aniline Chemical Co., Inc., N. Y.] to 1000 cc. absolute methyl alcohol [acetone free]). Let stand overnight and then add an additional 800 cc. of aged Wright's staining solution. Filter and use.

Buffer Solutions.*—1. Field and Le Fleming Method:⁴

Dissolve 0.85 gm. Na_2HPO_4 (anhydrous) (if $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ or $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ are to be used, quantity of salt must be recalculated) and 0.4 gm. KH_2PO_4 in 1 liter of distilled water. This solution should have a pH of 7 to 7.2. If a different pH is desired the quantity of the salts is changed according to Sorenson's buffer standards.

2. Wilcox and Logan's⁶ Method:

pH	Na_2HPO_4 (anhydrous) m/15 9.5 gm./liter	$\text{m/15 Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ 9.2 gm./liter	Distilled water
		or $\text{m/15 KH}_2\text{PO}_4$ 9.07 gm./liter	
7 0	61 1 cc.	38.9 cc.	900 cc.
7 2	72 0 cc.	28.0 cc.	900 cc.

Thin films should be fixed for two to five minutes in absolute methyl alcohol (acetone free) before staining with Giemsa. The fixed thin films and the unfixed thick films are then placed in a 2 per cent Giemsa staining solution (1 part stain to 49 parts neutral buffered water) for forty-five minutes. To stain thin films in thirty minutes, use a 3 per cent solution of stain. Dehemoglobinization of the erythrocytes and staining of the parasites in the thick film take place simultaneously.

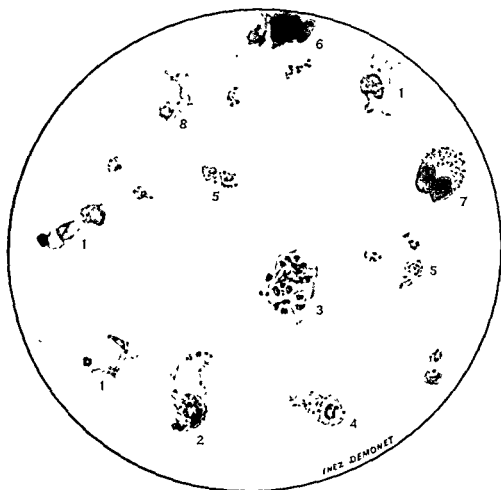
Upon removal from the stain, thin films should be dipped quickly two or three times in neutral buffered water. The thick films should be allowed to stand in neutral buffered water for three to five minutes for differentiation. The slides are then drained, but not blotted, and dried quickly. An electric fan is satisfactory to use, but heat is not advised.

Convenient containers for the staining solution may be obtained and kept on hand for use with various numbers of slides. For staining the quantity of slides that must be examined in malaria surveys, special staining racks holding 25 or more slides can be made. The method of Barber and Komp is also highly satisfactory. Inch squares of cardboard are placed between the slides to hold them apart at the ends opposite to the films and in this manner 25 or more slides may be held together in a block by a piece of heavy paper and rubber band. The block of slides is then placed upright in the staining solution for the required time.

A rapid method for staining thick films has been developed.³ Thick blood films are dried in an incubator at 37° C. for one hour or with an electric hand hair dryer. The Wright-Giemsa stain is diluted 1 to 9 with the neutral buffered water. The slides are stained for ten minutes in this solution. The scum which forms on the surface of the stain is flooded off with neutral water and the films are then allowed to stand for one minute in neutral buffered water. Dry and examine.

* NOTE These mixtures should be prepared each week or be protected from absorbing CO_2 which will change the pH.

PLATE VII



P. vivax—thick film

1. Ameboid trophozoites.
2. Schizont—2 divisions of chromatin.
3. Mature schizont.
4. Microgametocyte.
5. Blood platelets.
6. Nucleus of neutrophile.
7. Eosinophile.
8. Blood platelet associated with cellular remains of young erythrocytes.

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The stain, in both procedures, should be used only once. Careful attention must be paid to keep the glassware clean and to dilute the stain properly for the prevention of troublesome precipitation.

The stained thin blood film is the method of choice for studying the morphology of malaria parasites and for the novice to arrive at the differential diagnosis. Nevertheless, the thick film technic is necessary to find the organisms in low-grade infections and to save time when large numbers of films must be examined. Since it has been estimated that an individual can examine from ten to fifty times more blood in a thick film in a given amount of time, three to five minutes for the systematic examination of a thick film is considered ample for a skilled microscopist. This time is in great contrast to the twenty to thirty minutes for the systematic search of a thin film. Experience in thick film examination is necessary before an individual can make a reliable diagnosis of malaria. The method

morphology of the parasites slightly so that seen in a thin film. If the examiner

morphology of human plasmodia, a little practice with the thick films will soon lead to proficiency and accuracy in diagnosis. The method has so many advantages that it should be mastered and utilized by all individuals and laboratories called upon to make frequent or occasional examinations of blood for plasmodia.

III. Counting of Malaria Parasites.—In the control of induced malaria, and in clinical and epidemiological studies, quantitative knowledge about the concentration of parasites in the peripheral blood is essential. Since the live parasites are not visible in all their intracellular stages, counts must be made from stained films. Two methods are used to make such counts: (1) The expression of parasite number in some chosen unit of red blood cells, generally 10,000 erythrocytes, or (2) counting of the parasites in a given volume of blood stained by the thick film technic.⁴ Both methods have their uses, advantages, and disadvantages, but the desired result is obtainable by either technic.

1. Thin films are prepared (preferably on cover glasses) and stained by the usual technic. Ordinarily the parasites are counted in 500 to 1000 red blood cells and then the total number is expressed in terms of 10,000 erythrocytes. However, if the number of parasites is small, the blood sample to be counted should be larger, 2000 to 3000 red blood cells. To have no more than a 10 per cent error in the count, the use of the following formula will give the number of erythrocytes to be counted:

$N \text{ equals } 45.954 \frac{I-P}{P}$ with N equal to the number of red blood cells, P equals parasites per sample and I equals the sample unit (10,000).²

In using this method, care must be taken to systematically examine a swath across the width of the film. Parasitized cells are found in greater number near the margins of films on slides than in the center.

2. The following method is a modification of Earle and Perez (1932) and is quoted from Wilcox and Logan (Symposium on Human Malaria, 1941):⁴

"Five cubic millimeters of blood are taken in a special pipet and spread evenly over an accurately measured 3 x 15 mm. area on a clean slide. (This may be marked on the slide by ruling instruments having diamond

points set at the exact distances.) When dry the smear is stained like any other thick film. Preliminary to enumeration the microscope is calibrated as follows: A Howard disc with a ruled square is inserted in the ocular. One side of the large square is measured with a slide micrometer and the area of the field covered by the square computed in square millimeters. The number of fields to be counted in order to cover one square millimeter is ascertained by dividing one square millimeter by the area of one square microscopic field. For example, since 5 cubic millimeters of blood are deposited on 45 square millimeters of space, 5 divided by 45 equals .11, which is the portion of one cubic millimeter of blood spread over one square millimeter. Then also since 1 divided by 0.11 cubic millimeters equals 9 (the number of square millimeters carrying a blood volume equal to one cubic millimeter), the number of parasites counted in the required number of microscopic fields to equal one square millimeter is multiplied by 9 to obtain the number of parasites per cubic millimeter. The requisite number of fields is selected from different parts of the smear to make the count as representative as possible. If count runs high, fewer fields are examined and an estimation made for the required number of fields."

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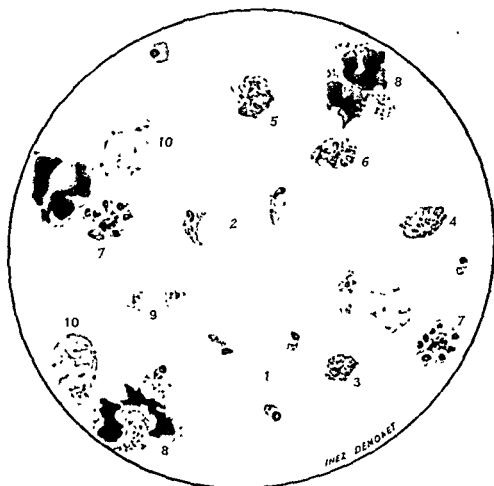
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DISSECTION OF ANOPHELINE MOSQUITOES FOR MALARIA PARASITES

Since no true prophylactic drug is known for the prevention of malarial infection, measures for the control of the disease have been directed against the transmitting vectors or anopheline mosquitoes which have a variety of different habits and modes of life. Not all anopheline mosquitoes in a given endemic area are responsible for transmission, in fact relatively few species are efficient vectors. Thus it is necessary to discover the species responsible for the spread of disease so that control measures can be concentrated intelligently and efficiently against the right species.

Information concerning mosquitoes and malaria in tropical and sub-tropical countries, where troops have to go, is frequently scant or non-existent. If the area involved presents a potential malaria problem, efforts should be made to determine the species of anophelines present during various seasons and the species that transmit malaria. The determination of species requires an entomologist or technician trained in the identification of mosquitoes, but the laboratory technician should know: (1) how

PLATE VIII



P. malaria—thick film

1. Small trophozoites.
2. Growing trophozoites.
3. Mature trophozoites.
- 4, 5, 6. Schizonts (pre-cgmenting) with varying numbers of divisions of the chromatin.
7. Mature schizonts.
8. Nucleus of leukocyte.
9. Blood platelets.
10. Cellular remains of young erythrocytes.

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to preserve and ship adult mosquitoes so that they may be identified, and (2) how to dissect an anopheline mosquito and examine it for malaria parasites.

I. Preservation and Shipping of Mosquitoes.—The ability to recognize an adult anopheline mosquito and determine its sex is assumed. (Reference to the entomological section of this manual will provide identifying characters.) Adult mosquitoes may be shipped alive in individual glass vials (8 x 1.5 cm.) to a nearby laboratory.¹ If vials are properly prepared with moist cotton in a one-hole cork, and a strip of blotting paper wedged inside along the length of the vial, mosquitoes will survive up to three days.

Shipping of killed mosquitoes is preferable because live infected mosquitoes may escape in a non-infected area and because wing scales needed for specific identification are better preserved. The anophelines are killed in a test tube or vial with chloroform fumes, exposing them for at least several minutes to definitely kill them. Individual mosquitoes are then carefully placed between layers of cotton, lens paper or other soft paper in a pill box (1½ inches in diameter by ¾ inch deep) or other suitable container so that they will be firmly held in place. Three to six mosquitoes are sufficient for one box. Crowding will only lead to the rubbing off of wing scales needed for identification. In the tropics or humid climates, the use of a few naphthalene or paradichlorobenzene flakes or a minute drop of creosote in each box will help preserve the insects. Labels containing collection data (locality, where caught, date, etc.) should accompany each box.

II. Dissection.—The fundamental purpose of dissecting wild female anopheline mosquitoes or anophelines experimentally infected is to determine the infection rate of plasmodial oöcysts on the stomach and of sporozoites in the salivary glands. Many methods are suitable for the procedure and skill is quickly obtained after a short period of practice.

Materials needed for dissection are a compound binocular dissecting microscope or dissection microscope with a simple lens, 7-20x magnification; a compound microscope; dissecting needles (straight surgical needles fixed in a handle and ground to a point or sharp blade); curved fine-pointed dissection forceps; cover glasses; microscopic slides; physiological saline solution (plain and tinted with methylene blue solution); Bunsen burner or alcohol lamp and filter paper.²

Each mosquito to be dissected is caught in a test tube and killed with chloroform just before the procedure is started. The legs and head are cut off, but the wings are kept intact. Place the mosquito, neck to the right, in a small shallow drop of tinted saline on the microscopic slide. A cover glass is held over the thorax as shown in Figure 72 and released. The thorax is held by the needle in the left hand. Gently press the cover glass with the forceps to press out the paired trilobed salivary glands as the thorax is pulled away from the cover glass. If the glands do not come out, they may be teased out of the thorax. Fat and debris may partially obscure the glands, but gentle raising and lowering of the cover glass will wash them free. Excess liquid may be drawn off with filter paper to facilitate examination.

To dissect the stomach, place the abdomen in a deeper part of the saline drop. Hold the mosquito by means of a needle in the right hand. With the needle in the left hand, nick the seventh and eighth segments

above and below. Then insert the point in the last segment and pull gently to draw out the stomach and Malpighian tubules. Cut off the tubules, sever the fore and hind guts close to the stomach and transfer

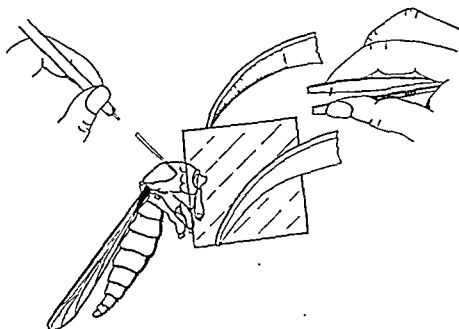


FIG. 72.—Method of holding needle, forceps and cover glass in the dissection of the salivary glands (Barber and Rice, courtesy of *Am. Jour. Hyg.*)

the stomach to a clean drop of saline. Add a cover glass and if the stomach is too contracted, warm the slide carefully to expand the organ for examination.

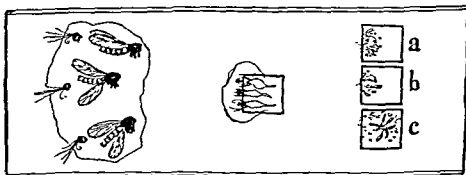


FIG. 73.—Arrangement of the slide preparations of the salivary glands and stomachs. (Barber and Rice, courtesy of *Am. Jour. Hyg.*)

the sporozoites or oöcysts. The sporozoites are slender fusiform bodies about 12 to 14 μ in length. They are not always identified easily so that making of a dry film and staining with Wright's or Giemsa's stain will

help verify the diagnosis. The chromatin mass in the center stains red and the cytoplasm stains blue. Wet fixation of films with Schaudinn's fluid plus 5 per cent glacial acetic acid and staining with Heidenhain's iron-alum hæmatoxylin is also satisfactory.

The fresh preparation of the stomach is examined with a similar lens combination to discover the presence of spherical or ovoid oöcysts which vary from 5 to 50 μ in diameter. Young oöcysts may be identified by the presence of unmistakable pigment granules which were carried along from the gametocyte stage. Mature cysts have a characteristic striated appearance caused by the arrangement of the sporozoites. The posterior end of the stomach usually contains the greater number of oöcysts and frequently staining procedures are needed to detect light infections or very young oöcysts.

Fixation and staining of stomachs as whole mounts may be performed without removing the cover glass from the fresh preparation or they may be fixed immediately after dissection and transferred from one solution to another in vials or watch glasses with a pipet.¹⁴ Schaudinn's fluid plus 5 per cent glacial acetic acid or modified Bouin's fixative (saturated aqueous solution picric acid 75 cc., formalin 15 cc., glacial acetic acid 10 cc., warm and add 1 gram of urea crystals; stir until dissolved) give satisfactory fixation in five minutes. Wash in 50 per cent alcohol for several hours or preferably overnight to remove fixative. Stomachs with oöcysts may be stored in 70 per cent alcohol. Before staining transfer to distilled water and then to a 1 to 10 dilution of Mayer's acid hemalum* for one hour. Remove and wash in 1 per cent solution of acetic acid three to five minutes, then neutralize in 1 per cent sodium carbonate (color changes to blue). Dehydrate by passing through ascending grades of alcohol, clear in carbol-xylene, then xylene and mount in gum dammar or clarite. Upon examination, the oöcysts are stained more darkly than the wall of the stomach.

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TECHNIC OF INDUCED MALARIA

The value of malaria as a means of inducing fever has been so widely and successfully practised that the technic for inducing this disease for therapeutic purposes is given in some detail. No advice concerning the selection of patients, or short-wave radio or by injection of protein such as the typhoid vaccine. Each of the methods have their purposes and value, but the use of induced malaria for the production of fever has been so widely and successfully practised that the technic for inducing this disease for therapeutic purposes is given in some detail. No advice concerning the selection of patients,

* Mayer's Acid Hemalum: dissolve 1 gm. of hematoxylin in 50 cc. of 90 per cent ethyl alcohol and warm. Add this solution to a solution of 50 gm. of aluminum potassium sulfate (dissolved by heating) in 1000 cc. of distilled water. Cool solution and filter. Add 2 cc. of glacial acetic acid to 100 cc. of hemalum solution.

clinical and laboratory findings in the malaria therapy of neurosyphilis can be included here. Only information pertinent to the choice of malaria species, inoculation, course, control, and termination of the infection is given.

I. Choice of Malaria Species.—The four species of human malaria parasites have been used for malaria therapy although benign tertian malaria (*P. vivax*) is the infection of choice for white patients and quartan malaria (*P. malarie*) for colored patients. Negroes exhibit a certain degree of natural resistance to *P. vivax* infection. Malignant tertian (*P. falciparum*) malaria can be used in both white and colored patients, but since it is pathogenic and highly invasive, control is more difficult and a constant check of parasite count is required for safeguarding the patient. Individuals coming for treatment from endemic malarious areas might be resistant to infection with certain strains because of acquired immunity. In such refractory cases a different species or strain should be utilized to produce infection.

Although indigenous naturally acquired strains can be used to induce malaria in paretics, strains of known clinical and therapeutic behavior have definite advantages for use.

The most widely used strains in this country include the McCoy, Cleveland and St. Elizabeth strains of *P. vivax*, the U. S. Public Health Service and Jones strains of *P. malarie* and the Long strain of *P. falciparum*.² If none of these strains or other strains carried along in large hospitals are available for use and it is necessary to employ a new strain, every effort should be made to detect the presence of *P. falciparum* because its inherent pathogenicity involves much greater risk as a therapeutic agent.

II. Inoculation of Patient.—Two methods can be used to induce malaria: (1) direct inoculation of infective blood, and (2) natural inoculation by the bite of infected anopheline mosquitoes. The first method is the most practical and easy to use, since the second method requires the breeding of mosquitoes in an insectary, a patient with sufficient gametocytes in the peripheral blood to infect mosquitoes, and then the feeding and maintenance of the insects under carefully controlled conditions for ten to thirty days to allow the completion of the sexual cycle and development of sporozoites in the salivary glands.

For direct inoculation, blood from a patient with a typical infection is drawn and injected into a recipient immediately, or defibrinated with glass beads or coagulation is prevented by the use of sodium citrate (1 cc. of a 2.5 per cent solution for every 10 cc. of blood). The amounts used for inoculating the recipient may vary from 1 to 10 cc. Unless larger amounts are used, blood typing is not generally necessary, although the use of matched blood whenever possible is to be preferred. Blood can be stored for future use at 4° C. (40° F.) in serum vials and will remain infective up to a period of two weeks. Special containers are needed for shipping blood so that parasites remain viable.⁴

The blood may be inoculated by intradermal, subcutaneous, intramuscular or intravenous routes. Intravenous inoculation is most commonly used and this method generally has the shortest incubation period. The incubation period is also dependent, however, on the number of parasites inoculated, the species of malaria and the natural resistance of the patient. When 5 to 10 cc. of infected blood are inoculated intravenously, the incuba-

tion period of tertian malaria is about seven days, quartan malaria ten to forty days or more and malignant tertian malaria about seven days. Storage or shipping of blood reduces the number of viable parasites and consequently will lengthen the incubation period. In every case a period of at least three weeks should be allowed for the development of infection before reinoculation. As stated previously, if the patient develops no malaria, another species or strain of a species should be given a trial.

Natural inoculation of patients with infected mosquitoes lengthens the incubation period and usually produces a more severe disease. Furthermore, the naturally induced infection is more difficult to control and terminate with antimalarial drugs. Sporozoites from macerated salivary glands of infected mosquitoes may be used to induce infection, but this procedure has similar drawbacks, besides being impractical for usage in most hospitals.

III. Course and Control of Infection.—Examination of the blood of patients inoculated by any of the above methods frequently reveals the presence of parasites one to several days before clinical symptoms begin. The thick film method is used for this examination and the information about appearance of parasites is valuable to the clinician. The early febrile reaction and paroxysms (100° to 101° F.) coincide with the presence of a variety of parasitic stages in the blood. After several paroxysms, the developmental cycle of the parasites becomes synchronous or "in step" and typical periodic paroxysms occur. It must be remembered that daily or quotidian paroxysms can be more common than the typical periodic paroxysms. In tertian malaria the typical paroxysm starts with a sharp shaking chill associated with a rapid rise in temperature to 104° to 106° F. The peak of temperature is maintained for one to three hours after which it falls gradually to normal. Profuse sweating occurs accompanied by malaise, severe headache, anorexia and exhaustion. Only those paroxysms are counted which produce a temperature of 104° F. or over.¹ The debilitating effects of daily paroxysms may require the temporary interruption of a course of malaria therapy. A single intramuscular injection of 0.2 gm. of sodium bismuth thioglycollate (thiobismol) will produce such interruption, permitting the patient to recuperate sufficiently to continue with his course of malaria. The thiobismol should be given not more than ten hours before the next febrile attack, so that it will take out one of the cycles of a double tertian infection or prevent one paroxysm in a single tertian infection. A respite from febrile attacks lasts about one to five days. This discovery of Schwartz,⁴ Cole *et al.*⁵ is a very important one and allows the continuation of malaria therapy with *P. vivax* in patients who could otherwise not withstand the severity of quotidian paroxysms. Unfortunately thiobismol appears to have little effect on *P. malarix* and *P. falciparum*.¹

No agreement exists among clinicians about the number of paroxysms necessary for optimum therapeutic results. Numbers varying from 8 to 20 are commonly permitted after which time the disease is terminated with suitable dosages of antimalarial drugs. Actually a number of conditions determine the time of terminating the disease. The physical condition of the patient, the intensity and effects of the febrile reactions play a rôle in making the decision. The lesser number of paroxysms is usually advocated in hospitals where no counts of parasites per cmm. are made. This procedure is unfortunate because a patient who can take nine paroxysms for

their beneficial therapeutic effect should not have the malaria terminated. The counting of erythrocytes, white blood cells and malaria parasites should be done at regular intervals and urinalysis should be carefully performed. When these vital laboratory tests are performed the optimum benefits and hence paroxysms from therapeutic malaria can be provided for the patient without undue risk. Overwhelming infections with parasites are detected when they occur and the severity of the developing anemia is known. High parasite counts and severe anemia along with unremitting pyrexia, rapidly enlarged and tender spleen, exhaustion, cardiac and renal disturbances, cyanosis, edema, convulsions, increased blood urea, development of another disease, severe jaundice and rapid debilitation are the principal indications for termination of the malaria.

Induced malaria, particularly tertian malaria, will frequently abort or terminate itself clinically before the desired numbers of paroxysms have occurred. In such cases, the parasites may persist in the peripheral blood for some time but different strains of the same species or quartan malaria can be reinoculated into white patients and malignant tertian in negroes to produce additional paroxysms.

No generalizations can be made about the treatment of induced malaria because of the variable reactions of strains to quinine and atabrine. Many strains being used for induced malaria are very susceptible to quinine but a safe procedure includes adequate treatment of the infection and even of spontaneously terminating cases to prevent relapses and persistence of the parasites as latent malaria. A safe treatment is 10 grains of quinine three times a day for four days followed by 10 grains a day for eight weeks. For quinine resistant strains atabrine in 0.1 gm. amounts three times a day for five days is satisfactory. In case a patient with *P. falciparum* is to be treated, a course of plasmochin to sterilize the gametocytes should follow the quinine or atabrine.

Every effort should be made to keep patients in screened wards until the infection has been cured. Statements have been made about strains of plasmodia losing their ability to produce gametocytes after direct inoculations from patient to patient over a long period of time. Unless a competent malariologist has examined infected blood for gametocytes on several occasions, the safest procedure is to confine all patients in efficiently screened quarters where no anopheline mosquitoes can reach them. Of course, in places such as the northern United States where no anopheline mosquitoes breed during the winter months, confinement to screened quarters is necessary only during the breeding season in the summer.

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IMMUNOLOGICAL REACTIONS IN MALARIA

Within recent years, significant advances have been made in our knowledge of immunity in malaria. Immunity in this infection has been little understood, and has even been questioned, but the results achieved offer new encouragement and methods of approach for an eventual solution of unsolved problems. Practical application of certain immunological tests, which have been worked out experimentally, has been attempted on a small scale. For that reason, the following tests are outlined to provide an approach, if nothing more, to the future development of less complicated and precise procedures.

Studies on human, simian and avian malaria have proved that immunity is acquired during the course of an infection. The persistence of the acquired immunity is apparently dependent upon the presence of parasites in the body, and is thus variously named "premunition" or "infection immunity." Antibodies which are produced can be detected by complement fixation, agglutination and precipitation reactions. Skin tests have not been successful.

Cellular reactions are also a part of the immune response. Proliferation and activation of the lymphoid-macrophage system for increased phagocytosis begins very soon after the initial paroxysm of the infection. The great increase or hyperplasia of reticulo-endothelial cells in the spleen, causes the progressive enlargement of that organ or splenomegaly. Apparently the cellular immunity of the host is not independent of humoral immunity, but the two interact to determine the degree of protection acquired by the host during the course of the disease. Infection and order to obtain some
sease.

against the infecting strain of parasite, failing to protect against a heterologous strain of the same species. Evidence for this has been obtained from studies with induced human and experimental simian malaria. The existence of strains of each species of parasite helps to explain the variable virulence of the same species from different countries and also the immunity of a native population to local strains and susceptibility to strains from another area.

I. Complement-fixation Reaction.—In 1938, the complement-fixation reaction was placed on a practical basis.^{1,6} The simian parasite, *Plasmodium knowlesi*, produces an overwhelming infection in the *rhesus* monkey and hence, a rich source of parasites for making antigen became available for the first time. Antigens of *P. knowlesi*, *P. vivax*, *P. malariae* and *P. gallinaceum* of the domestic chicken have now been made and used successfully. The antigen, made of *P. knowlesi*, is group specific, binding complement in the serum of monkeys with a chronic homologous infection

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Studies on sera from induced human malaria and from patients in a

hyperendemic malarious area have served to demonstrate and verify the specificity of the reaction.^{4,5} The test becomes positive approximately two weeks after the initial paroxysm and persists for about five months after circulating parasites disappear from the peripheral blood. Relapses cause a rise in titer, indicating that circulating parasites and thus a source of antigen are necessary to maintain the titer at a significant level.

Preexisting syphilis does not interfere with the specificity of the reaction. Absorption with the syphilitic reagin of serum from positive blood fails to affect the reaction.³ The reverse is not true, however, for it is well known that sera from patients with malaria will give false positive Wassermann and Kahn tests.⁷

II. Other Tests.—Agglutination,⁸ precipitin,¹⁰ and precipitative² tests are species specific reactions in contrast to the group specificity of the complement-fixation test. Difficulties in preparing antigens of the human plasmodia have prevented the practical application of agglutination and precipitin tests. The greater sensitivity of the precipitative technic has overcome some of the difficulties and these methods deserve further trial.

Recent work has shown that sporozoites from the salivary glands of infected mosquitoes will agglutinate to a high titre in the serum of an experimental animal or a patient infected with the homologous species.⁹ The sporozoites are potent antigens and have high agglutinogenic properties. This method might have practical diagnostic value, if quantities of infected mosquitoes could be provided for dissection in samples of suspected sera.

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THE HÆMOFLAGELLATES OF THE BLOOD AND TISSUES

Prior to the present conflict, human leishmaniasis and trypanosomiasis were diseases that were seen rarely only in hospitals of our large cities where foreign-born individuals came for medical attention. These diseases assume new importance now that men of our armed forces travel through or are stationed in countries where the above diseases are endemic. Studies on the etiology, clinical course, treatment and control of these diseases have provided one of the fascinating chapters in tropical medicine. Many medical and public health problems remain to be solved before morbidity

and mortality caused by these infections can be reduced significantly. Nevertheless, methods of diagnosis and treatment are being improved and each officer responsible for the diagnosis of parasitic diseases should become acquainted with the parasitology of these infections.

I. Classification.—Parasitic protozoa of the class Mastigophora, which live in the blood and tissues of man, and in various vertebrate and invertebrate hosts are grouped in the family Trypanosomidae Doflein 1901.

There are six genera as follows:

Genus *Leptomonas*.—The life cycle contains leishmania and leptomonad forms, which occur only in invertebrates. Infective cysts are formed and passed in the feces of the host.

Genus *Crithidia*.—The life cycle contains leishmania, leptomonad and crithidial forms, which occur only in invertebrates. Transmission is similar to *Leptomonas*.

Genus *Herpetomonas*.—The life cycle contains leishmania, leptomonad, crithidial and trypanosome forms which occur only in invertebrate hosts. Transmission is similar to *Leptomonas*.

Genus *Leishmania*.—The life cycle contains only leishmania and leptomonad forms, which occur in both a vertebrate and an invertebrate host. Transmission by the invertebrate to the vertebrate or *vice versa*.

Genus *Phytomonas*.—The life cycle contains only leishmania and leptomonad forms, which occur in both an invertebrate and plant host.

Genus *Trypanosoma*.—The life cycle contains leishmania, leptomonad, crithidial and trypanosome forms which occur both in an invertebrate and a vertebrate host. The occurrence of the four forms is true only for *Trypanosoma cruzi*. No leishmania or leptomonad forms are known for *T. gambiense* and *T. rhodesiense*. Transmission is by the invertebrate to the vertebrate or *vice versa*.

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ment, some confusion occurs when the name of the form taken by a parasite is used as a descriptive rather than a generic term. The following description of the various stages should clarify the confusion.

1. Stages in Life Cycle.—(a) *Leishmania Form*.—This stage is round or ovoid, seldom exceeding 3 to 5 μ in diameter. No flagellum or undulating membrane is present. Stained specimens reveal a centrally located vesicular nucleus, the densely staining kinetoplast or rod-like parabasal body and the blepharoplast. These stages occur primarily in endothelial cells and macrophages in infected animals or man. They may also develop *in vitro* in old cultures.

(b) *Leptomonad Form*.—This stage possesses a flagellum arising from the anterior blepharoplast. The body may be long or short and spindle-shaped. The nucleus is central in position. These stages develop in cultures or in insects when leishmaniform stages are inoculated or ingested.

(c) *Crithidial Form*.—The parabasal body and blepharoplast are formed slightly anterior to the central nucleus in this stage, giving rise to a short undulating membrane and occasionally a free flagellum. These stages may develop in cultures, in the alimentary tract of transmitting insects or in tissues.

TABLE 85.—HAMOTLAULATES OF MAN—THE DISEASES PRODUCED AND STAGES OF PARASITES FOUND

Species	Name and type of disease	Individuals infected	Lesions	Stages of parasite		
				Man	Insect	Culture
<i>Leishmania donovani</i>	Kala-azar, visceral infection	All ages	Spleen, liver, bone marrow, skin, etc.	Leishman-Donovan bodies	Flagellates (Leptomonads)	L.-D. bodies and flagellates
<i>L. infantum</i>	Mediterranean or infantile kala-azar, visceral infection	Children, Mediterranean, adults, So Am.	same	same	same	same
<i>L. tropica</i>	Oriental sore, cutaneous infection	All ages	Skin and lymph glands	same	same	same
<i>L. brasiliense</i>	So Am. leishmaniasis, cutaneous and mucocutaneous infection	All ages	Skin, lymph glands and mucocutaneous tissues of nose, throat and mouth	same	same	same
<i>Trypanosoma cruzi</i>	Chagas' disease, blood and tissue infection	Children, and few adults	Heart, glands and skeletal muscles	Trypanosome in blood, L.-D. bodies in tissues	Critthidia, metacyclic trypanosomes	Critthidia, metacyclic trypanosomes
<i>T. gambiense</i>	African sleeping sickness, blood infection	All ages	Nerve tissue and glands	Trypanosomes	Critthidia, metacyclic trypanosomes	?
<i>T. rhodesiense</i>	Rhodesian sleeping sickness, blood infection	All ages	Nerve tissue and glands	Trypanosomes	Critthidia, metacyclic trypanosomes	?

(d) *Trypanosome Form*.—In this form, the kinetoplast is located in the posterior end of the organism, where it may be terminal or subterminal. The undulating membrane originates from the blepharoplast and extends along the margin of the body, being composed of a marginal axoneme and cytoplasm which attaches the membrane to the body. The axoneme extends anteriorly to form a free flagellum.

The shape, size, and the general morphology may vary during the cyclic development of the organisms in transmitting insect vectors. These stages or "metacyclic forms" eventually produce infective stages which transform into the typical adult trypanosome.

2. *Morphological versus Physiological Species*.—The majority of protozoa described in the preceding chapters can be distinguished from each other by morphological characters. The human species of *Leishmania* and *Trypanosoma* of African sleeping sickness are so similar in structure that morphological differentiation is impossible. Consequently, other characters such as the disease produced, susceptibility of animals, immunological and cultural behavior, and infectivity of insect vectors, are used to distinguish species. In Table 85, the species which cause the diverse disease entities are tabulated to aid in arriving at a specific diagnosis.

DIAGNOSIS OF LEISHMANIASIS

Leishmaniasis is not a single entity but includes three types of diseases (Fig. 75), differentiated according to the organs or tissues affected. The visceral infection or kala-azar, caused by *Leishmania donovani*, occurs in parts of India, China, and Africa. Infantile kala-azar, caused by *L. infantum*, is found in countries bordering the Mediterranean Sea. Cutaneous infection called Oriental Sore (synonymous with Bouton d'Orient, Delhi boil, Aleppo boil) is caused by *L. tropica*. South and Central American leishmaniasis is a cutaneous infection in certain areas, and both a cutaneous and muco-cutaneous disease in other areas. The cutaneous disease is called uta, chiclero's ulcer, forest yaws, buba and by other diverse local names, and the muco-cutaneous disease is called naso-pharyngeal leishmaniasis or espundia. The organism, *L. brasiliensis*, causing these of the viscerotome of endemic areas, Commission which studied the disease created a new species, *L. chagasi*, for the etiological agent of the infection, but the organism has now been identified with *L. infantum* of infantile kala-azar.

I. *Kala-Azar*.—The parasites, *L. donovani* and *L. infantum*, are taken up by the reticulo-endothelial cells in which they multiply by binary fission. The parasitized cells become enlarged (Fig. 74, No. 1) and eventually rupture to free the organisms in the plasma or tissue fluids (Fig. 74, No. 2), which in turn are taken up again by phagocytic cells. The infection induces extensive proliferation of endothelial cells, monocytes and macrophages, particularly in the spleen, lymphatic glands, liver and bone marrow. Splenomegaly and enlargement of the liver develop in practically all infections, facts which are useful in diagnosing the disease, if malaria is excluded. Leishman-Donovan bodies may be found in the oral and nasal secretions, urine and feces. The disease may run an acute, subacute or chronic course,

and it may become epidemic. Mortality in untreated cases may reach 90 per cent, with secondary infections being responsible for a high percentage of deaths.

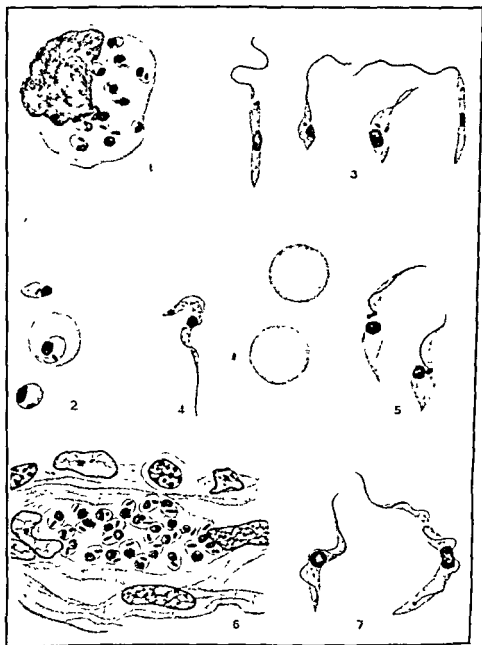


FIG 74 — Hemoflagellates (X 2000).

1, Mononuclear leukocyte containing *Leishmania brasiliensis*; 2, free Leishman-Donovan bodies, *L. brasiliensis*, with one organism superimposed on a red blood cell; 3, various stages found in the culture of *L. brasiliensis*; 4, adult trypanosome of *T. cruzi*; 5, cultural or crithidial forms of *T. cruzi*; 6, an aggregate of aflagellate *T. cruzi* in heart muscle; 7, large and small adult trypanosomes of *T. gambiense*. (Original)

Infantile kala-azar differs mainly by degree from the adult disease. Secondary infections are usually the cause of mortality. Dogs are now

believed to be the primary reservoir of *L. infantum* for transmission by species of blood-sucking insects, *Phlebotomus* or sandflies.

Although the results of extensive research over a period of years pointed to the transmission of kala-azar and Oriental Sore by species of *Phlebotomus*, crucial experiments have now proven that *Phlebotomus argentipes* (sandfly) transmits the infection in India,^{4,5,6} and that *P. papatasi* can transmit cutaneous leishmaniasis.¹

II. Diagnosis.—Since kala-azar may assume a variety of clinical forms, diseases such as malaria, schistosomiasis, Banti's disease, typhoid, paratyphoid, ng at a correct diagnosis demonstration of the which are dependent upon the biochemical changes of the blood are helpful too.

1. Demonstration of the Parasites.—Although the parasites multiply within the reticulo-endothelial cells, they are not always demonstrable in the peripheral blood by the direct examination of films stained with Wright's or Giemsa stain. The use of culture media for the detection of infection is most likely to succeed. Approximately 10 cc. of blood are taken and added to a solution of sodium citrate in physiological saline. After centrifugation at a speed to pack and separate the erythrocytes from the leucocyte layer or buffy coat, inoculate a few drops of the buffy coat into two to four tubes of NNN medium. Incubate at 22° to 28° C. and examine carefully from the tenth to the twentieth day for motile flagellates (Fig. 74, No. 3) which develop from the aflagellate *Leishmania* inoculated.

2. Spleen or Liver Puncture.—This test is performed to obtain and demonstrate the Leishman-Donovan bodies by direct examination of stained films made from the extracted splenic or liver pulp, or by inoculation of NNN medium with pulp in order to grow out the parasites by cultivation.

This procedure requires care and precision because of the danger of rupturing the enlarged spleen or liver in the process. The method and technic of Napier³ (see also Manson-Bahr and Strong) has been used with great success. The operation is successful when pulp and not blood is withdrawn, because the parasites are in the tissue cells. Sternal puncture, to obtain bone marrow, is also used by some clinicians to demonstrate parasites. If there are no contraindications for the operation, the spleen is the organ of choice because of the available numbers of parasites found there. Stained pulp is positive for Leishman-Donovan bodies in about 90 per cent of patients with kala-azar, but it is advisable to divide the sample withdrawn into one part for staining and one part for cultivation in order to pick up infections in which few parasites are present.

3. Biochemical Tests.—Physiological changes in the serum of patients with kala-azar have been the basis for the development of useful diagnostic tests. The aldehyde test or formol-gel test (Napier) and the antimony test (Chopra) are the most widely used.

(a) *The Aldehyde Test.*—One cc. of clear serum from the patient is placed in a small test tube (Wassermann tube) and 1 drop of formaldehyde is added. Shake well and place in rack. If the serum becomes viscid immediately and gels in a minute or two or up to twenty minutes, becoming whitish and opalescent, the reaction is diagnostic of kala-azar. Milkiness

LEISHMANIASIS

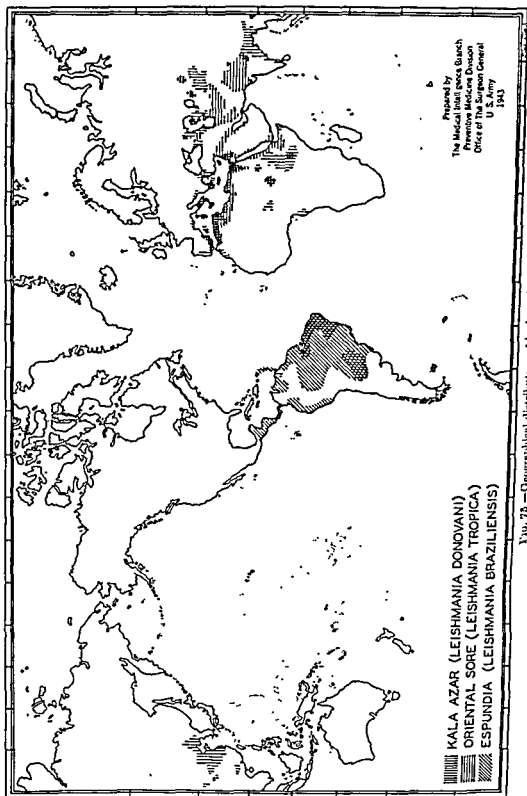


FIG. 78.—Geographical distribution of leishmaniasis.

of the serum without the formation of a gel may occur in serum from a case of kala-azar of less than three months duration but the result should be considered doubtful without the demonstration of parasites. If the serum gels but does not become opaque, the reaction is again doubtful. Normal sera remain fluid and clear and should always be run as controls.

(b) *Antimony Test (Chopra).*—This test is based on the formation of a heavy precipitate in serum from a patient with kala-azar when a 4 per cent solution of pentavalent antimony compound is added. Whole serum or serum diluted 1 to 10 with distilled water is placed in a small tube using a capillary pipet. With a clean pipet, add a 4 per cent solution in distilled water of a pentavalent antimony compound (urea-stibamine or stibosan) so that the solution comes to lie below the serum. A heavy coarsely flocculent precipitate forms when the antimony solution comes in contact with positive sera. In a very early case, the precipitate may not appear for ten to fifteen minutes. The flocculum is very difficult to disperse and persists for more than twenty-four hours. This test compares favorably with the aldehyde test for the detection of infection.

III. *Cutaneous and Muco-cutaneous Leishmaniasis.*—These infections, caused either by *L. tropica* or *L. brasiliensis*, are characterized by the development of an indurated papule which ulcerates. The demonstration of the parasites in stained cells scraped from the margin of the ulcer is relatively easy during the early stage. Just as soon as ulceration occurs and enlargement begins, secondary infection with bacteria develops and it becomes increasingly difficult to demonstrate the presence of *Leishmania*. The older ulcers usually have an irregular margin which is indurated. The parasites are found in the large mononuclear leucocytes and macrophages of the indurated margin, with multiplication of *Leishmania*, rupture of cells, and phagocytosis progressing in a manner similar to the spread of infection in the spleen of a patient with kala-azar. Regional lymphatics may become involved but the infection is generally a local one with no parasites circulating in the peripheral blood. Diagnosis of the infection depends upon the demonstration of parasites in stained films made from the crater of an early lesion or from material withdrawn from the indurated margin by means of a sterile fine capillary pipet. Cultures can be made successfully with scrapings of early lesions or with the material withdrawn from the indurated margin by means of a capillary pipet or hypodermic needle. The ulcer should be thoroughly washed with sterile saline solution and the unbroken skin of the indurated margin should be cleansed with a mild antiseptic solution. A number of culture tubes (NNN) should be inoculated because of the great danger of bacterial contamination, since leishmania will seldom grow in the presence of bacteria. Cultures should become positive in uncontaminated tubes in from ten to twenty days.

Every effort should be made to demonstrate *Leishmania* in a suspected lesion. Although the indurated margin of a leishmania ulcer is characteristic, ulcers caused in the tropics by fungi, spirochetes and a variety of bacteria may confuse the diagnosis. Biopsies of margins of ulcers (when lesions are not on face) for sectioning and staining are sometimes helpful in making a diagnosis.

Cases of muco-cutaneous leishmaniasis or espundia give a history of having had a primary lesion at the nasal orifice or on the lips before the involvement of the mucosa of the nose, throat or mouth began. Demon-

stration of the parasites is very difficult in these cases because of their long duration and consequent organisms before coming for treatment from those of yaws (gangosa) and granulation tissue in the nose or throat for demonstration of the parasites and pathology is frequently of aid in diagnosis. If the patient comes from an area where espundia occurs, a diagnosis on clinical grounds and a therapeutic test may be necessary if the parasites cannot be demonstrated.

IV. Cultivation of *Leishmania*.—The cultivation of *Leishmania* for the diagnosis of visceral leishmaniasis has definite value. The diagnosis of cutaneous and muco-cutaneous leishmaniasis by cultural methods is less successful because of difficulties in preventing bacterial contamination which prevents the persistence and development of the organisms. The formulæ for useful media are given below. For diagnostic purposes, the NNN medium is very satisfactory, but for experimental purposes and for the production of a vaccine, other media which give heavier growth are preferred.

1. Blood Agar or NNN Medium (Novy, MacNeal & Nicolle, 1908).—

Stock agar

Agar	14 gm.
Sodium chloride	6 gm.
Distilled water	900 cc.

Dissolve and dispense in tubes or flasks in amounts convenient for use. Before using add one-third volume of sterile defibrinated rabbit's blood to the dissolved agar cooled to 45° to 50° C. Mix thoroughly, tube to make a long slant with no butt. After agar is set, the addition of sterile rubber stoppers will help maintain water of condensation which prolongs life of culture. Test for sterility by incubation for twenty-four hours at 37° C.

Inoculate material for cultivation, in water of condensation and on slant. Incubate for best growth at 20° to 25° C. Transfers should be made every twenty to thirty days for maintenance.

2. *Leptospira* Medium (Noguchi, 1924).—

Physiological saline (0.9 per cent)	500 parts
Fresh rabbit serum	100 parts
Nutrient agar (2 per cent, pH 7.2)	100 parts
Rabbit hemoglobin solution	10-20 parts
(1 part defibrinated rabbit's blood in 3 parts distilled water. Centrifugalize and use clear supernatant fluid)	

Tube and test for sterility before using. The amount of hemoglobin solution may be increased to give better growth of *Leishmania*. The organisms grow in a layer near the surface of the medium and should be subcultured approximately every thirty days.

3. Adler's Modification (1926) of Noguchi's *Leptospira* Medium.—

Agar	1 part
Locke's solution containing 0.2 per cent dextrose	8 parts
Fresh rabbit serum	1 part

This medium is satisfactory for the cultivation of species of *Leishmania* and *Trypanosoma cruzi*.

1. Modified Sallo and Schmidt Medium (Cleveland and Collier, 1920).²—

Veal infusion	50 cc
(50 gm. Bacto-veal, Difco 1000 cc. distilled water)	
Protose peptone (Difco)	10 gm
Sodium chloride	5 gm
Distilled water	550 cc

Dissolve, adjust reaction to pH 7.1 and autoclave. Add 20 cc. of 50 per cent glucose (sterilized by filtration or in autoclave at 10 pounds pressure for ten minutes) and 60 cc. of horse cells laked with 2 parts of distilled water. Dispense the medium in tubes or flasks. Cultures with this medium are very rich in growth and long lived.

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DIAGNOSIS OF TRYPANOSOMIASIS

I. **General.** Species of trypanosomes are common organisms, parasitizing the blood and tissues of vertebrate hosts. The non-pathogenic trypanosomes, which are the most common, feed not only on man but on a wide variety of animals and manatees of distinct economic importance, because of the disease they produce in domestic and wild animals (Table 86). Three species produce disease in man. *Trypanosoma brucei* cause of West African sleeping sickness, *T. brucei* cause of Rhodesian sleeping sickness and *T. cruzi* of Chagas' disease in South and Central America (Fig. 70). African sleeping sickness is transmitted by various species of tsetse flies of the genus *Glossina*, the most important species being *G. morsitans*, *G. palpalis* and *G. tachinoides*. Chagas' disease is transmitted by blood sucking hemipterous insects of the genera *Triatoma* and *Reclinorhina*. In addition a species of insects have been found naturally infected and used in experimental transmission of the disease.

II. **African Sleeping Sickness.** Two distinct clinical pictures are produced by *T. brucei* and *T. rhodesiense*, but the exact stage of the disease can only be determined on the basis of laboratory tests. The early stage of the disease in man and cattle is closely related to that of the animal. The pathologic changes are a marked enlargement of the lymphatic glands, enlargement of the spleen, liver, and kidneys, and a moderate leukopenia. The blood picture is a leukopenia with a relative increase of lymphocytes and a decrease of polymorphonuclear cells (Fig. 74, A, C, E). The characteristic stage of the disease is the "sleeping sickness" stage, which is characterized by a marked enlargement of the lymphatic glands, enlargement of the spleen, liver, and kidneys, and a moderate leukopenia. The blood picture is a leukopenia with a relative increase of lymphocytes and a decrease of polymorphonuclear cells (Fig. 74, A, C, E). The characteristic stage of the disease is the "sleeping sickness" stage, which is characterized by a marked enlargement of the lymphatic glands, enlargement of the spleen, liver, and kidneys, and a moderate leukopenia. The blood picture is a leukopenia with a relative increase of lymphocytes and a decrease of polymorphonuclear cells (Fig. 74, A, C, E).

African sleeping sickness, caused by *T. rhodesiense*, has the more rapid course of the two infections and consequently is more severe in its manifestations. In both infections, however, transmission results from the bite of an infected tsetse fly which mechanically carried the organism or introduced infective "metacyclic" trypanosomes at the time of taking a blood meal. The incubation period is from two to three weeks after which time trypanosomes may be found in the peripheral blood. Infection of the blood stream is rarely heavy and extreme difficulty may be experienced in demonstrating the parasite in the blood, either by the use of thick films or fractional centrifugation. Swelling of the lymphatics, particularly the posterior cervical glands, is a characteristic early symptom. Puncture of swollen lymphatic glands during the acute febrile period, withdrawal of glandular juice and examination of several preparations for motile trypanosomes is one of the most successful diagnostic procedures. When patients begin to show involvement of the central nervous system in the advanced stage of the disease, examination of cerebrospinal fluid may give positive results. The fluid is withdrawn and centrifugalized. Trypanosomes, if present, may be demonstrated in the sediment. An increased cell count and globulin content of the spinal fluid are also indications of invasion of the central nervous system by the parasites.

If the above methods fail to establish the diagnosis, susceptible laboratory animals, such as the white rat, guinea pig, dog, and monkey may be inoculated. Two to 10 cc. of blood are withdrawn from the patient, and amounts, in proportion to the size of the animal, are inoculated intraperitoneally. Since these primary inoculations of a foreign host may fail, negative results with this test should not be considered absolutely diagnostic.

Nothing has been said about the use of cultivation procedures because methods that

T. gambiense a:
in vitro indicat.
ments. The methods given below have not been used to any great extent, but the results reported indicate that the methods deserve further trial.

1. Brutsaert and Henrard's Medium.¹—

<i>Solution A.</i> Ringer's solution for invertebrates	
NaCl	6.50 gm.
KCl	0.14 gm.
CaCl ₂	0.12 gm.
Distilled water to make 1000 cc	
<i>*Solution B.</i> Tyrode's solution	
NaCl	8.00 gm.
KCl	0.20 gm.
CaCl ₂	0.20 gm.
MgCl ₂	0.10 gm.
NaH ₂ PO ₄	0.05 gm.
NaHCO ₃	1.00 gm.
Glucose	1.00 gm.
Distilled water to make 1000 cc	

Both solutions are sterilized by filtration and are then distributed in culture tubes in the proportion of 2 cc. of Solution A and 2.5 cc. of Solution B. Add 2 cc. of citrated human blood (1 per cent citrate) and incubate at 37° C. for twenty-four hours, to determine sterility. Tubes should be kept in refrigerator and may be used up to two weeks after preparation.

In a syringe containing 1 cc. of Roche fluid (1 per cent solution of sodium polyanethol sulfonate) withdraw 5 cc. of the patient's blood. Distribute 0.5 cc. in each of 10 culture tubes and incubate at 25° to 28° C. Trypanosomes, if present in the inoculum, should be demonstrable in ten to twenty days after inoculation. The value of this medium for diagnostic purposes when other methods fail has been confirmed by Hawking.²

2. **Cultivation in the Developing Chick Embryo.**—This well-known method for propagating infectious agents has been utilized for species of trypanosomes by Longley, Clausen, and Tatum.⁶ Fertile hen's eggs, incubated eight to ten days, are prepared for inoculation by boring one hole into the air sac and another directly over the embryo or by the commonly used window technic. Sterile technic must be used throughout the operation. The inoculum, 0.5 cc. of material containing the trypanosomes, is injected into the allantoic cavity. Seal the holes in the eggshell or the window with a cover glass and incubate at 37° C. Heavy infection of the blood develops, resulting in the death of the embryo in four to five days. A variety of species, *T. rhodesiense*, *T. equiperdum*, *T. brucei*, *T. evansi*, and *T. hippicum*, have been successfully cultivated in developing embryos. The method is a valuable one for experimental purposes, but whether or not it will be useful for diagnosis of human infection will depend on results obtained.

III. **Chagas' Disease.**—Human infection with *Trypanosoma cruzi* of Chagas' disease is confined to South and Central American countries. The disease was originally discovered in Brazil (Chagas, 1909), but endemic foci are now known to exist in Argentina, Chile, Uruguay, Panama, Guatemala, and Mexico. Infected vectors have been found in the southwestern United States, but no human cases of the disease have been described.

Transmission of the disease takes place when the person being bitten scratches the infective "metacyclic" trypanosomes, which were defecated by the reduviid bug, into the wound. The resulting infection, which has an incubation period of ten to thirty days, consists of circulating adult trypanosomes in the peripheral blood and the invasion of tissue cells with transformation into leishmania forms, identical in morphology with those found in cases of kala-azar. The leishmania forms (Fig. 74, No. 6), 2 to 4 μ in diameter, divide by binary fission, eventually causing destruction of the

dependent upon the pathology produced by invasion and destruction of tissue. Practically every organ in the body may be invaded, but the parasites are found most often in heart, muscle, and the neuroglia cells of the central nervous system.

The diagnosis, as in many parasitic infections, depends primarily upon the discovery and identification of the adult trypanosomes or leishmani-form stages of *T. cruzi*. Adult trypanosomes measuring about 20 μ in length (Fig. 74, No. 4), are most apt to occur in the peripheral blood during acute febrile attacks. Long, slender and short, stumpy forms occur. Since they are never numerous, even in the acute stage of the disease, great difficulty may be experienced in demonstrating them during the chronic stage.

Three methods are of value for the demonstration of adult *T. cruzi*: (1) examination of a "thick film" (prepared and stained by the same

TRYPANOSOMIASIS

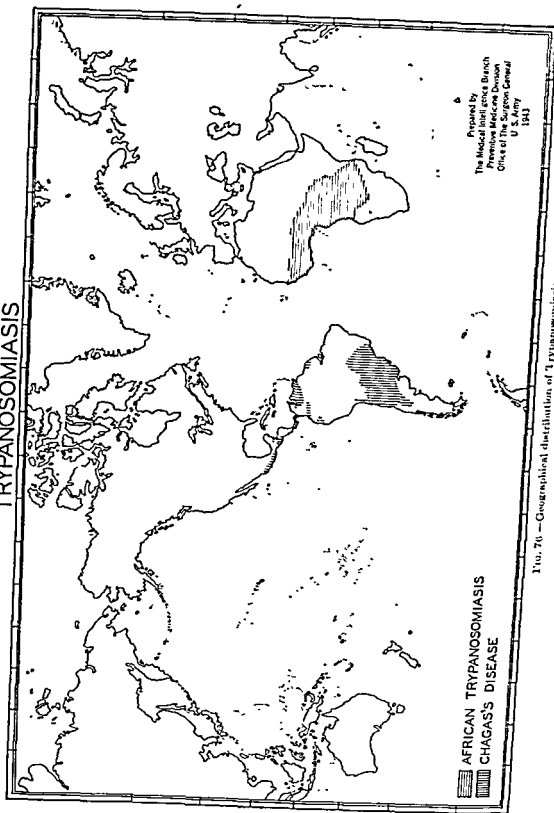


FIG. 76.—Geographical distribution of trypanosomiasis.

method used for the diagnosis of malaria); (2) inoculation of a young guinea pig or young dog with blood; and (3) the xenodiagnostic test (Brumpt, 1914) which consists of allowing parasite-free laboratory-reared triatomids to feed on the patient. Examination of the feces or intestinal contents of these insects after eight to ten days will reveal trypanosomes, if they were present in the blood of the patient. The "thick film" method is the most practical because a number of films can be examined over a period of days during the febrile attack, in an effort to discover the light infection of trypanosomes. Biopsies of swollen lymph nodes, or spleen and sternal punctures have been used in an effort to demonstrate tissue stages. Since visceral leishmaniasis occurs in South America, the discovery of tissue stages should be followed by cultivation of blood and biopsy tissue in order to exclude leishmaniasis.

The media of choice for cultivation are NNN medium and Kelser's medium:⁴

1. Kelser's Medium.—

Bacto-Beef (Difco)	2 5 gm.
Bacto-Peptide (Difco)	12 5 gm.
NaCl	3 5 gm.
Distilled water	500 0 cc.

Dissolve the Bacto-Beef in the water by placing the mixture in a water bath at 55° C. for one hour. Add other ingredients and dissolve by placing flask in boiling water for five minutes. Filter through cotton until clear and then adjust reaction to pH 7 with 1 N solution of sodium hydroxide. Measure total volume and add 1 per cent Bacto-Agar. Dissolve and dispense either 5 cc. to each test tube or 10 cc. to each small flask. Sterilize in autoclave under 12 pounds pressure for thirty minutes. Medium can be stored in this manner for later addition of dextrose and blood, or used immediately after adding 5 per cent of a 1 per cent sterile solution of dextrose (0.25 cc. to each tube or 0.5 cc. to each flask) and 5 per cent of fresh sterile defibrinated guinea pig blood. (The author has substituted defibrinated rabbit and sheep blood successfully.) After thorough mixing, tubes are slanted with a deep butt and short slant. Sterile rubber corks may be used to prevent evaporation. Incubate to test for sterility.

cultures can be made successfully at intervals of six to eight weeks. If water of condensation in tubes or flasks becomes low at any time, a dextrose-broth mixture (1 part of a 1 per cent sterile dextrose solution to 2 parts peptone broth) may be added.

Cultivation of *T. cruzi* is relatively easy on the medium given above. Flagellate forms or crithidial (Fig. 74, No. 5) and trypanosome forms develop in these cultures. The temperature for incubation is 22° to 25° C. and growth takes place on the slant and in the water of condensation. Subculture is necessary only every six to eight weeks and tubes frequently remain viable three months or more. Tissue cultures have also been successful⁵ and the author has obtained successful growth using the surviving tissue culture method devised by Zinsser, Wei and Fitzpatrick⁷ for the growth of Rickettsiae. Finally, the complement-fixation reaction as

TABLE 80—PRINCIPAL TRYPANOSOMES OF MAN AND ANIMALS*
Group A
Electrophoretic not "Terminal"

Species	Length	Definitive host	Intermediate hosts (vectors)	Geographical distribution	Susceptible laboratory animals	Culture	Diagnosis
<i>T. leish</i>	25 μ	Rats	Lucas (<i>Glossophylla</i> <i>crassa</i> , <i>Polyz</i> <i>crassa</i> , <i>Crematophylla</i> <i>farinosa</i> , <i>C. farinosa</i> , <i>Leishmania</i> <i>crassa</i>)	Cosmopolitan	White rats (in vivo, with difficulty)	NNN 25° C	None
<i>T. theileri</i>	60 μ to 70 μ	Cattle	Tsetse flies (<i>Tsetse</i> <i>gambosus</i> , <i>T. gambosus</i> , <i>T. gambosus</i>)	Cosmopolitan	None	Bouillon with blood, NNN 25° C	None
<i>T. melophagum</i>	50 μ to 60 μ	Sheep	House fly (<i>Melophagus</i> <i>ornatus</i>)	Temperate zones	None	Bouillon with blood, NNN with glucose 30° C	None
<i>T. theodori</i>	50 μ (?)	Horses	House fly (<i>Leptopoda</i> <i>caprina</i>)	Pakistan, Syria	None	Bouillon with blood and dextrose 30° C	None
<i>T. cruzi</i> (and related species)	20 μ	Man, armadillo, cat, opossum	"Kissing bug" (<i>Triatoma</i> <i>neglecta</i> , <i>T. infestans</i> , <i>T. sordida</i> , <i>Triatoma</i> <i>chagasi</i> , <i>Leishmania</i> <i>protrix</i> , <i>Erathyrus</i> <i>capitatus</i>)	South America, including Panama (Brazil, Venezuela)	Guinea pigs, in vivo, rats, rabbits, dogs, cats, monkeys	NNN 25° C, Tissue culture, Heber's medium	Glassa' diagnosis

Group B <i>Electrophoretic not "Terminal" or "Subterminal"</i>						
Species	Length	Definitive host	Intermediate host	Geographical distribution	Susceptible laboratory animals	Diagnosis
<i>T. evansi</i> (and related species)	18 μ to 31 μ	Horses, mule, ass, cattle, buffalo, sheep, elephant, dog	Horse flies (<i>Tabanus</i> and <i>Stomoxys</i>) (mechanical)	Cosmopolitan, tropics and subtropics	Hate, rabbit, guinea pig, dog	Developing chick embryo

	25 μ to 28 μ	Horse, ass	None, transmitted during coitus	Europe, India, Africa, America	Dogs, rabbits, mice, rats, guinea pigs, monkeys, sheep	Same	Dourine
<i>T. equiperdum</i> Monomorphous free flagellum	18 μ to 26 μ	Cattle, sheep, goat, horse	Tsetse fly (<i>Glossina palpalis</i> , <i>G. morsitans</i> , <i>G. tachinoides</i> , <i>G. longipalpis</i>)	Tropical Africa	Rabbits	None	Souma
<i>T. vivax</i> Monomorphous free flagellum	18 μ to 32 μ	Cattle, sheep, horse	Tsetse fly (<i>G. morsitans</i> , <i>G. brevipalpis</i>)	Tanganyika, Nyasaland	None	None	Usually fatal
<i>T. evansi</i> Monomorphous free flagellum	12 μ to 16 μ	Cattle, sheep, horse	Tsetse fly (<i>G. palpalis</i>)	Uganda, Congo	None	None	Usually fatal
<i>T. congolense</i> Monomorphous free flagellum but free	9 μ to 18 μ	Cattle, horse, sheep, goat, pig, ass, dog	Tsetse fly (<i>G. palpalis</i> , <i>G. morsitans</i> , <i>G. tachinoides</i> , <i>G. brevipalpis</i>)	Tropical Africa, Zanzibar	Rats, dogs (with difficulty)	Ponselle's medium 25° C	Usually fatal
<i>T. sinu</i>	14 μ to 24 μ	Monkey, pig, horse, sheep	Tsetse fly (<i>G. morsitans</i> , <i>G. palpalis</i> , <i>G. pallidipes</i> , <i>G. tachinoides</i> , <i>G. brevipalpis</i>)	Tropical Africa	Monkey, no small animals	None	Fatal in monkeys
<i>T. brucei</i> Polymorphic	12 μ to 35 μ	Domestic mammals (wild animals are reservoirs)	Tsetse fly (<i>G. morsitans</i> , <i>G. palpalis</i> , <i>G. pallidipes</i> , <i>G. tachinoides</i> , <i>G. brevipalpis</i>)	Tropical Africa	All except monkeys	NNN plus glucose 32° C. Ponselle's medium at 25° C (difficult) Brutsaert and Hendrick's medium, 25° C.	Nagana
<i>T. theileri</i> Polymorphic	12 μ to 35 μ	Man (reservoir antelope)	Tsetse fly (<i>G. morsitans</i> , <i>G. palpalis</i> , <i>G. a. yunnanensis</i>)	Last Africa (Rhodesia, Nyasaland, Tanganyika, Mozambique)	Same as <i>T. brucei</i>	Same as <i>T. brucei</i> and developing chick embryo	Sleeping sickness (acute)
<i>T. gambiense</i> Polymorphic	15 μ to 30 μ	Man (reservoir antelope)	Tsetse fly (<i>G. palpalis</i> , <i>G. f. f. f.</i> , <i>G. brevipalpis</i> , <i>G. pallidipes</i> , <i>G. tachinoides</i>)	Tropical Africa West Coast 15° N to 15° S East Coast 10° N to 10° S	Same as <i>T. brucei</i> but less virulent	Same as <i>T. brucei</i>	Sleeping sickness (chronic form)

* Adapted from Hoare, C. A., and Coutelen, F., *Ann. Parant. hum et comp.*, 11, 196-200, 1933.

The nucleus is stated under T braces

f No posterior nucleated forms when in la-

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devised by Kelser⁴ is an extremely valuable test to diagnose chronic infections and even discover latent cases of the disease.⁵ The antigen is prepared from *T. cruzi* grown in the special medium devised by Kelser for this purpose.

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PART VII

Helminthology

CHAPTER XXXVII

HELMINTHIC INFECTIONS

By ERNEST CARROLL FAUST

DEFINITION AND OUTLINE OF GROUPS OF HELMINTHS

HELMINTHIC infections are produced by helminths or parasitic worms. These are many-celled organisms belonging to the phyla Nemathelminthes (literally "thread worms") and Platyhelminthes (literally "flatworms"), and, in addition, to the classes Acanthocephala (literally "thorny-headed worms") and Hirudinea (leeches).

I. *Nemathelminthes*.—The Nemathelminthes or roundworms are elongated cylindroidal worms, covered with a relatively thin resistant cuticle which constitutes the essential body support; usually tapering at both ends; unsegmented but possessing a longitudinal axis, a body cavity and a complete digestive tract. The sexes are usually separate. The worms vary in size from microscopic objects to forms measuring a meter in length. Many species are free-living in water or mud; some are parasitic on or in plant tissues; many others parasitize animal hosts, including man. Some forms have a simple life cycle; others have a complicated mode of development. Most of the species belong to the class Nematoda ("threadlike body") but a few belong to the class Gordiacea (so-called "horse-hair worms").

1. *The Nematoda or True Roundworms*.—The more important species of true roundworms parasitizing man include the following: *Ascaris lumbricoides* (giant intestinal roundworm); *Trichocephalus trichiurus* (synonym, *Trichuris trichiura*, whipworm); *Necator americanus* ("American" hookworm); *Ancylostoma duodenale* ("Old World" hookworm); *Ancylostoma braziliense*; *Strongyloides stercoralis*; *Enterobius vermicularis* (pinworm, seatworm, human "oxyuris"); *Trichinella spiralis* (trichina worm); *Wuchereria bancrofti* (Bancroft's filaria); *W. malayi* (Malay filaria); *Onchocerca volvulus* (convoluted filaria); *Loa loa* (loa worm), and *Dracunculus medinensis* (Medina or dragon worm). In addition, there are many so-called "rare species" which are common parasites of domestic and wild animals but are occasionally (although incidentally) parasites of man.

2. *Gordiacea*.—The Gordiacea are probably never true parasites of man, although on occasion mature or immature specimens of this group get into the mouth and are later vomited or passed in feces.

II. *Platyhelminthes*.—The Platyhelminthes or flatworms are usually elongated worms which are flattened dorsoventrally, have a longitudinal

axis and possess no body cavity. The spaces between visceral organs in this group are occupied by loose, relatively undifferentiated parenchymatous tissue. Most of the species are hermaphroditic. A few forms have a simple life cycle but some have a very complicated mode of development. The class Turbellaria comprises almost exclusively free-living species, while the Cestoidea (tapeworms) and Trematoda (flukes) are exclusively parasitic.

1. The Cestoidea or Tapeworms.—The adults of these worms typically

eggs). A digestive tract is lacking. The stages in their development consist of egg, embryo, larva, and adult worm (technically cestode). The adult worm constitutes a completely independent organism. The species which parasitize man include: *Diphyllobothrium latum* (fish tapeworm); *D. mansoni* (Manson's tapeworm); *Hymenolepis nana* (dwarf tapeworm); *H. diminuta* (rat tapeworm); *Dipylidium caninum* (dog tapeworm); *Tænia saginata* (beef tapeworm); *T. solium* (pork tapeworm), and *Echinococcus granulosus* (hydatid worm).

2. The Trematoda.—The adult trematode responds to a simple life cycle, consisting of two acetabula, and a blind one more posteriorly placed on the venter of the worm. The life cycle is very complex in all of the species parasitizing man and consists of at least three generations which may be represented diagrammatically as follows:

(a) First generation: egg → larva (miracidium) → first generation sporocyst (or redia).

(b) Second generation: Second generation sporocyst (or redia), produced in man.

(c) Third generation: The third generation sporocyst (or redia) produces the miracidium and the cercaria are free-living in water, but between these two stages there is a required parasitic existence within certain species of snails or other molluscs to which the flukes are specifically adapted. The two-fold multiplication within the snail may amount to as great an increase in progeny as 250,000 to 1.

The more important species which parasitize man include: *Schistosoma*

sinensis (Chinese liver fluke), and *Paragonimus westermani* (Oriental lung fluke). In addition, there are several other species of flukes which incidentally parasitize man.

III. Others.—1. Acanthocephala.—The class Acanthocephala was for many years regarded as a group of the phylum Nematelminthes, but is now known to have nearer kinship to the Cestoidea (tapeworms). Two species of this class, *Macracanthorhynchus hirudinaceus*, a parasite of hogs,

and *Moniliformis moniliformis*, a parasite of rats, have on rare occasions been recorded as parasites of the human digestive tract.

2. *Hirudinea*.—The *Hirudinea*, or leeches, constitute a class group of the phylum *Annelida*, the most familiar of which is the common earthworm.

IMPORTANT DATA ON HELMINTHS PARASITIZING MAN, INCLUDING STAGES MOST USEFUL FOR DIAGNOSIS

I. *Ascaris lumbricoides* (common large roundworm of the intestine).—This worm typically lives in the lumen of the small bowel; it is cosmopolitan

3, especially
n diameter,

sexes have three conspicuous fleshy lips surrounding the mouth, one median dorsal and two ventrolateral. The males are curved somewhat ventrad at the posterior end. Infection is acquired from ingesting fully embryonated eggs picked up from soil; the larvæ hatched from eggs in the small bowel require a lung journey before developing to adults in the small intestine. Migrating larvæ may produce atypical pneumonia; adults give rise to gastro-intestinal upsets and nervous disorders, at times with edema and frequently with eosinophilia. The worms may produce acute intestinal obstruction, appendicitis, biliary duct obstruction, peritonitis, etc. The females lay about 200,000 eggs per day; these are passed in feces. *Diagnosis* is made by recovering fertile or infertile eggs in the stool (Fig. 77, A, B), or adult or immature worms may be passed in the stool or evacuated spontaneously from the anus, mouth or nares.

II. *Trichocephalus trichiurus*, synonym: *Trichuris trichiura* (human whipworm).—This worm lives with its anterior end basted into the mucous coat of the cecum, appendix, distal segment of the ileum or ascending colon. It is cosmopolitan in distribution but most common in moist warm countries. The worms have a delicate anterior three-fifths and fleshy posterior two-fifths to their body. The males are 30 to 45 mm. long with the fleshy posterior end coiled like a watch spring; the females, 35 to 50 mm.

attached and develop to adults in about ninety days. Light infections produce mild intestinal disturbances and nervous disorders; heavy infections may simulate hookworm disease, with microcytic hypochromic anemia, neutropenia, eosinophilia, and severe emaciation. The female lays a few thousand eggs per day; these are passed in feces. *Diagnosis* is made by recovering typical eggs in the stool. (See Fig. 77, I.)

III. *Necator americanus* (so-called "American" hookworm).—This worm lives attached by its head capsule to the mucous coat of the small bowel, most frequently near the jejunal-ileal junction. It is distributed throughout the warm climates of the Western Hemisphere and the warm climates south of 20° N. latitude in the Eastern Hemisphere. The worms have a distinct buccal capsule provided with two dorsal and two ventral cutting plates and a pair of internal lancets; the males are 7 to 9 mm. long by 0.3 mm. in diameter, with an umbrella-like copulatory bursa surrounding

the posterior end and a pair of copulatory spicules fused distally and terminating in a barb; the females are 9 to 11 mm. long and 0.4 mm. in diameter, with a pointed posterior end.

IV. *Ancylostoma duodenale* (so-called "Old World" hookworm).—This worm is prevalent in the "Old World" north of 20° N. latitude. It has a buccal capsule provided with two pairs of distinct teeth on its "upper" aspect. The males are 8 to 11 mm. long by 0.4 to 0.5 mm. in diameter; the females, 10 to 13 mm. long by 0.6 mm. in diameter. Epidemiological and clinical aspects are essentially similar to *Necator americanus*.

V. *Ancylostoma braziliense*.—This species has a "spotted" distribution throughout warm moist climates. Its buccal capsule is smaller than that of *A. duodenale* and the inner pair of teeth are much smaller. The males are 7.75 to 8.5 mm. long by 0.35 mm. in diameter; the females, 9 to 10.5 mm. in length by 0.37 mm. in diameter. Human, canine and feline strains are physiologically distinct; in man human strains produce intestinal infection but canine and feline strains cause only "creeping eruption" within the deeper layers of the skin, due to failure of infective-stage larvæ to enter blood vessels. *Diagnosis* in intestinal infections is based on recovery of

LEGEND FOR FIG. 77

worm or seatworm), with completely developed larva, passed in feces or more usually deposited by the mother worm on the perianal or perineal skin; D, *Ancylostoma duodenale* ("Old World hookworm") or *Necator americanus* ("American hookworm"), early cleavage stage, passed in semi-formed feces; E, *A. duodenale* ("Old World hookworm") or *N. americanus* ("American hookworm"), with completely developed first-stage (rhabditoid) larva passed in constipated stool or developed in feces that have stood twenty-four to forty-eight hours in the laboratory; F, *A. duodenale* ("Old World hookworm") or *N. americanus* ("American

worm), with unsegmented embryo, usually with bile-stained outer shell, passed in feces;

in feces, X, *Schistosoma hæmatobium* (vesical blood fluke), with developed miracidium, passed in urine or (rarely) in feces, Y, *Schistosoma mansoni* (Manson's blood fluke), with developed miracidium, passed in feces; Z, *Schistosoma japonicum* (Oriental blood fluke), with developed miracidium, passed in feces

R, S, T, and U, $\times 666$, all other figures $\times 333$. (Faust's Human Helminthology.)

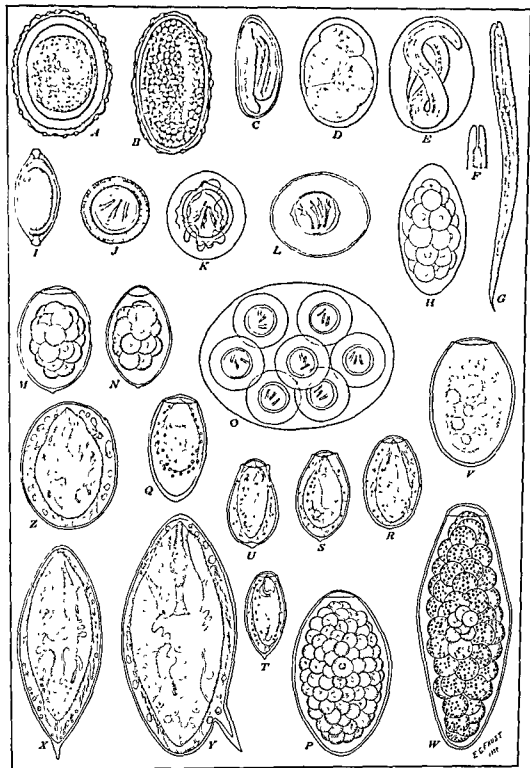


FIG. 77

the eggs in stools; in cutaneous infections on the characteristic linear tunnels in the skin.

Hookworm infection is acquired from contact of the skin with moist soil containing the filariform-stage larvæ, which penetrate to the peripheral blood capillaries, are carried in the blood stream to the lungs, are coughed up, swallowed and develop to maturity after attachment to the mucosa of the small bowel. Light infections produce little or no symptoms; heavy infection causes "hookworm disease," characterized by microcytic hypochromic anemia, frequently with anoxia and tachycardia, neutropenia, eosinophilia, dysphagia, severe malnutrition and emaciation, and frequently dysfunction of endocrine organs. The females lay a few thousand eggs each per day; these are passed in feces. *Diagnosis* is based on recovery of eggs (or occasionally larvæ) from stool. (Figs. 77, D, E, F, and 78, A.)

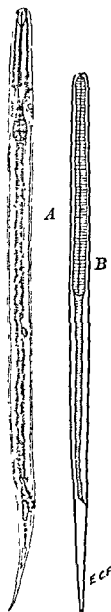
VI. *Strongyloides stercoralis*.—The parasitic female worm lives embedded in the mucous coat of the bowel, most commonly at the level of the duodenum. This worm occurs usually in warm moist climates. The parasitic male is transient, and is seldom recovered; the parasitic female is filiform, 2.2 mm. long by 30 to 75

out in feces. Infection is acquired and migration through the lungs proceeds as in hookworm infection. Strongyloidiasis usually causes a mucous diarrhea, sometimes with severe dehydration; with a high eosinophilia, at first a neutrophilic leukocytosis, later a neutropenia and monocytosis, at times secondary anemia; in chronic cases neurotoxic manifestations sometimes develop. The females lay several hundred eggs each per day. *Diagnosis* is based commonly on the recovery of rhabditoid larvæ (Fig. 77, G) in freshly passed stool, or possibly filariform larvæ (Fig. 79, B) or free-living adults in older stools.

FIG. 78.—A, Filariform larva of hookworm, with acuminate tail; B, filariform larva of *Strongyloides stercoralis*, with "notched" terminus of tail. A, $\times 160$ (after Faust, adapted from Looss); B, $\times 120$ (after Faust).

VII. *Enterobius vermicularis* (pinworm or seatworm, "oxyuris").—Mature worms are attached to the mucous coat of the cecum and appendix, but gravid females typically wander out the anus and migrate on the perineal skin, frequently entering the genital tract of female

patients. The infection is cosmopolitan in distribution but more common in certain family and institutional groups than in the population at large. The males are 2 to 5 mm. long by 0.1 to 0.2 mm. in diameter, with a ventrally curved posterior end; the females, 8 to 13 mm. long by 0.3 to 0.5 mm. in diameter with a long, delicately pointed posterior end. Infec-



tion is acquired by swallowing fully embryonated eggs picked up by fingers after scratching contaminated perianal or perineal skin or from soiled underclothing or other fomites. Enterobiasis may be essentially symptomless, may cause an appendicopathia, intense pruritus perian et

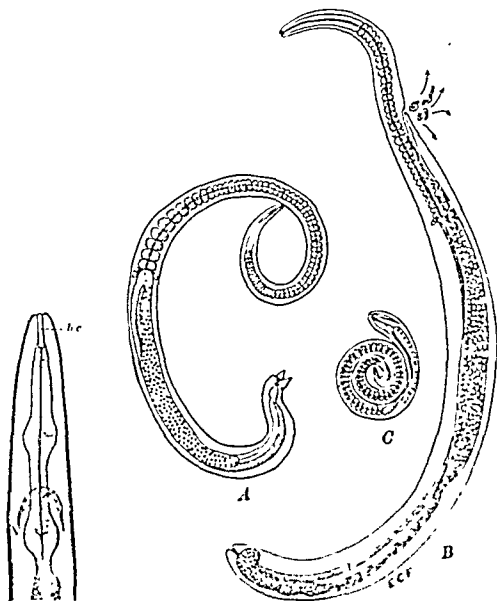


FIG. 79

FIG. 80

FIG. 79 — Anterior end of *Trichostrongylus axei*, with long narrow buccal chamber (bc) and intermediate esophageal portions as well as posterior bulbus of muscular esophagus. $\times 400$. (From Faust's Human Helminthology, after Sanderson.)

FIG. 80 — *Trichostrongylus axei*. A, Adult male, $\times 40$; B, adult female, $\times 40$; C, larva, $\times 60$. (From Faust.) A, B, after Yecko and May, *Intestinal Parasites of Veterinaries*, courtesy of J. and A. Churchill, Ltd.; C, adapted from Butler.

perini, or severe nervous disorders, at times nycturia or syncope. Diagnosis is based on the recovery of typical eggs (fig. 77, C), recovered in 5 to 10 per cent of patients in the study, but much more commonly from swabbing of the perianal skin with cellulose or cellophane tape.

VIII. *Trichinella spiralis* ("trichina" worm).—The adults live in the duodenal mucosa, the encysted larvæ in skeletal muscle. (See Figs. 80, 81.) The infection is cosmopolitan in populations eating pork which is not thoroughly cooked or otherwise properly processed. The males are 1.4 to 1.6 mm. long by 40 to 50 microns in diameter; the females, 3 to 4 mm. long by 60 to 75 microns in diameter. Infection is acquired from eating infected raw pork. The larvæ excest in the duodenum, enter the mucosa and rapidly develop to adults. The females, described as "cysticercoid," are found in the venules and lymphatics, are encysted in the muscle and are found out in skeletal muscle and encysted.

less or may have a sequence of symptoms characterized by acute food poisoning, excruciating myositis with edema, myocarditis and severe neurotoxic manifestations; it is usually accompanied by high eosinophilia. *Diagnosis* is difficult in the early stages but after the twenty-first day it may be made by muscle biopsy or by the intradermal test with specific *Trichinella* antigen.

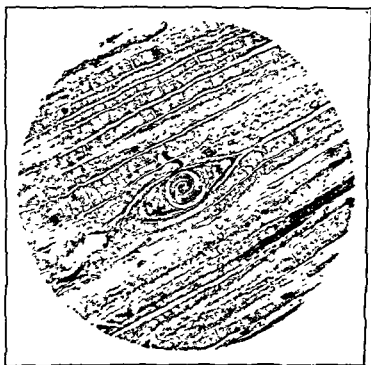


FIG 81 — Encysted trichinella larvæ in striped pork muscle. (Aldridge, Am Jour. Med Sci)

IX. *Wuchereria bancrofti* (Bancroft's filaria) and *W. malayi* (Malay filaria).—These are filaria worms with a predilection for development as adults in the small lymphatic vessels and in lymphoid tissues. They have an extensive distribution throughout warm climates (*W. bancrofti* practically engirdling the world, *W. malayi* from Southern China to the Dutch East Indies and up the Bay of Bengal). The males are 40 mm. long by 0.1 mm.

FILARIASIS
(WUCHERERIA BANCROFTI)



Prepared by
The Medical Intelligence Branch
Preventive Medicine Division
Office of The Surgeon General
U. S. Army
1943

These microfilariae are picked up by certain mosquitoes, transform into larvæ in the thoracic muscles and then migrate down the mosquito's proboscis sheath. Infection results from the bite of the infected mosquitoes, with an incubation period in man of approximately one year. The incuba-

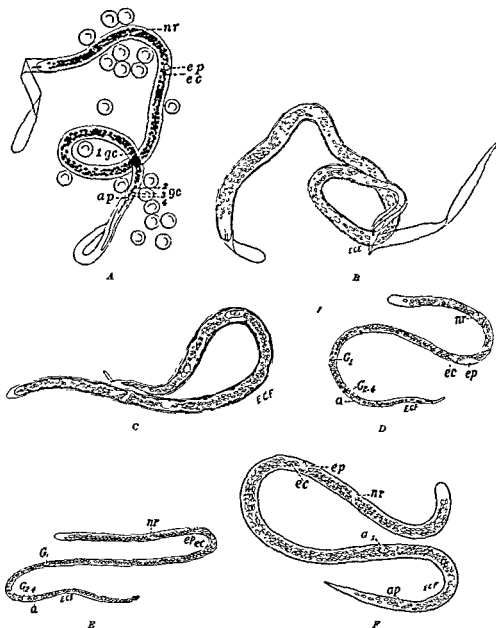
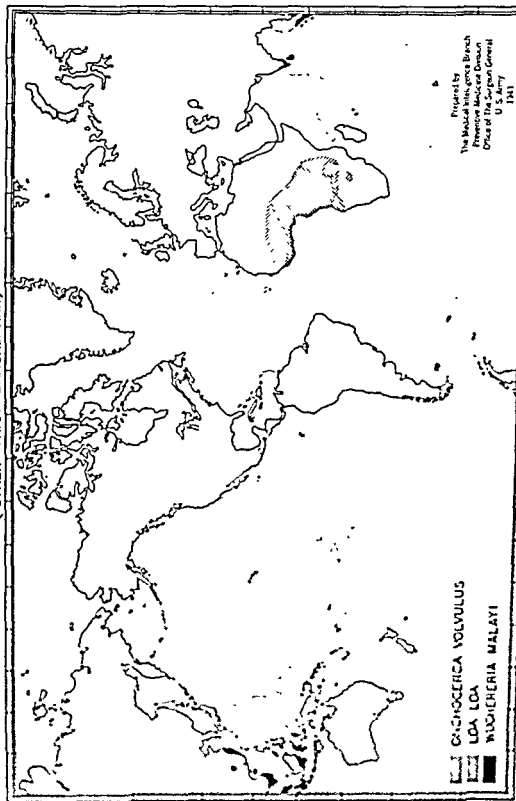


FIG. 83.—Microfilariae of important or common human filaria worms. A, *Microfilaria* of *Wuchereria bancrofti*, B, of *Wuchereria malaya*; C, of *Loa loa*; D, of *Mansonella ozzardi*; E, of *Acanthocheilonema perlati*; F, of *Onchocerca volvulus*. nr, nerve ring; ep, excretory pore; ec, excretory cell. 1, 2, 3, 4 gc and G₁, G₂₋₄, so-called "genital cells;" a or ap, anal pore. A, B, C, "sheathed," D, E, F, "unsheathed." A-E, from peripheral blood; F, from biopsied skin. All $\times 532$. (From Faust's Human Helminthology.)

tion period and post-incubation period may be essentially symptomless, but are usually followed by episodes of acute inflammatory reaction in and around involved tissues and lymphatic vessels, especially when parent worms begin to die; with chronic sequelæ of elephantiasis, varicose groin

FILARIASIS
(OTHER THAN BANCROFTIAN)



glands, chyluria, chylocele, etc. *Diagnosis* is based on the recovery of microfilariae in blood films or other appropriate body fluids, and their specific identification (Fig. 83, A, B.)

X. *Onchocerca volvulus* (convoluted filaria).—This worm lives as an adult in the midst of a dense subcutaneous fibrous nodule. It has a wide distribution in Central America, especially in the region of Guatemala and

escape from the parent nodule and migrate in cutaneous and subcutaneous lymphatic capillaries and intercellularly, with a tendency to swarm towards the eyeball or optic nerve, producing lesions which lead to diminished vision and frequently to blindness. The life cycle involves a small blood-sucking gnat, *Simulium*, which is the source of infection for other human beings. *Diagnosis* is based on recovery of a particular type of "unsheathed" microfilaria (Fig. 83, F) from biopsied skin placed in warm physiological salt solution, as well as demonstration of adult worms in section of subcutaneous nodules removed by operation.

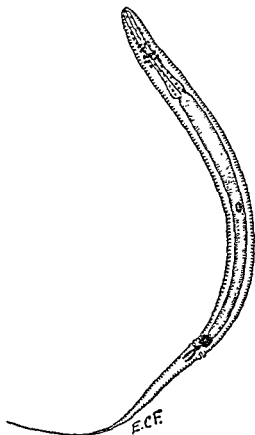


FIG. 85.—Rhabditoid larva of *Dracunculus medinensis*, as discharged by gravid female worm from ruptured cutaneous blister into fresh water. (From Faust's Human Helminthology)

XI. *Loa loa* (loa worm).—The adults migrate through the subcutaneous tissue and periodically cross the front of the eyeball. This infection is

just behind the path of the worm. It is acquired from bites of an infected mango fly, *Chrysops*, which is the required intermediate host

XII. *Dracunculus medinensis* (Medina or dragon worm).—The males and young females develop in the viscera or deeper somatic tissue; the gravid females migrate to the skin, producing a blister and discharging their larvæ when the blister bursts on contact with fresh water. This infection is widely distributed throughout India, the Near East and Africa. It is acquired from accidentally swallowing the infected intermediate host, water "fleas" (*Cyclops*), in raw drinking water. *Diagnosis* is based on prodromal allergic manifestations, together with the characteristic track and opening of the gravid female worm, and her partial emergence and discharge of larvæ (Fig. 85) in contact with fresh water.

XIII. *Diphyllobothrium latum* (the fish tapeworm).—The adults are attached by their scolex to the mucous coat of the small intestine. This infection is extensively distributed throughout Siberia, North, East and Central Europe, in the lake districts of northern Michigan, Minnesota and adjacent Canada, and in minor foci elsewhere. The adult worm measures 3 to 10 meters in length, grooves, a "neck" and the latter discharging m:

The eggs require a period of embryonation in fresh water before hatching. When ingested by water "fleas" (*Diaptomus* or *Cyclops*), the ciliated embryos penetrate into the hemocele of this host and develop into first-stage larvæ; when the "fleas" are eaten by fresh-water fishes the larvæ develop to the second stage (*sparganum*) in the flesh of the fish. Human infection results from eating raw fresh-water fish. It may be symptomless or may cause digestive upsets, and at times may be associated with a macrocytic anemia and eosinophilia. *Diagnosis* is based on recovery of typical eggs (Fig. 77, M) in the patient's feces.

XIV. *Sparganum mansoni* (larval stage of *Diphyllobothrium mansoni*).—Mature larvæ of *D. mansoni*, naturally occur in many species of vertebrates; human infection, particularly in the Dutch East Indies, Indo-China. *Diagnosis*

is based on recovery of the living sparganum (about 6 mm. long, unsegmented) from the human body at operation or at autopsy.

XV. *Hymenolepis nana* (dwarf tapeworm).—The adults are attached by their scolex to the mucous coat of the small bowel. This infection is widely distributed in warm climates, especially among children. The adult worm measures 25 to 40 mm. in length, and consists of a scolex with four suckers and rostellar hooklets, a "neck," and not more than 200 proglottids, discharging mature eggs from the disintegrating distalmost proglottid. The eggs passed in the stool are immediately infective for man if they reach the mouth and are swallowed. Infection may produce neurotoxic manifestations. *Diagnosis* is based on recovery of the specific type of egg in feces (Fig. 77, K).

XVI. *Hymenolepis diminuta* (rat tapeworm).—The adults are attached by their scolex to the mucous coat of the small bowel. This infection is cosmopolitan in its distribution in rats and mice but is rather uncommon in man. The adults are 20 to 60 mm. long and consist of a scolex with four suckers but no rostellar hooklets, a "neck," and a thousand or more proglot-

ng distalmost proglottids, intermediate host, as they develop into a larval stage. The infected flea is accidentally taken into the mouth and swallowed. Infection in man may be accompanied by mild to severe neurotoxic symptoms. *Diagnosis* is based on the recovery of characteristic eggs (Fig. 77, I) in the patient's stool.

Dipylidium caninum (dog tapeworm).—The adults are attached to the mucous coat of the small intestine. Infection is cosmopolitan in its distribution in dogs, less common in cats, and is occasionally found in human beings, particularly children. The adult worm measures 25 to 100 cm. in length and consists of a scolex with four suckers and a rostellar proboscis armed with several rows of hooklets, a "neck," and a series of several hundred immature to gravid proglottids. The distalmost proglottids break off from the chain, either individually or in groups, and

pass in feces. The proglottids contain larvæ of the flea, which develop into the flea's hemocele. These larvæ survive the flea's metamorphosis to the adult stage and provide the source for canine, feline or human infection when the infected flea is accidentally taken into the mouth and is swallowed. Infection may occasion digestive upsets and nervous disorders. *Diagnosis* is based on the discovery of evacuated proglottids and, on their compression or dissection, the detection of many mother embryonic groups, each containing several characteristic eggs (Fig. 77, O).

XVIII. *Tænia saginata* (beef tapeworm).—The adults are attached by their scolex to the mucous coat of the small bowel; they measure 5 to 25 meters in length and consist of a scolex with four suckers and hooklets, a "neck" and a series of several hundred proglottids, provided with 15 to 20 main longitudinal uterine stems, become detached from the parent worm individually or in groups and migrate out the anus or are passed in feces. The eggs are not usually found in feces but are discharged from disintegrating proglottids on the soil. They require ingestion by cattle to hatch, the embryos then migrate to the ox's striated muscle, where they develop into bladder worms (*cysticercus* larvæ). Infection is acquired from eating infected raw beef. This infection may result in severe digestive disorders, with diarrhea, dysphagia, loss of weight, insomnia and other neurotoxic manifestations, or even acute intestinal obstruction; it is frequently accompanied by a secondary anemia, eosinophilia and neutropenia with moderate monocytosis. *Diagnosis* is based on the demonstration of gravid proglottids with their characteristic number of main lateral uterine arms, each packed with *Tænia* eggs (Fig. 77, J), which cannot be distinguished from those of *T. solium*.

XIX. *Tænia solium* (pork tapeworm).—The adults are attached by their scolex to the mucous coat of the small bowel. The larvæ (*Cysticercus cellulosæ*) are also capable of parasitizing all organs and tissues of the human body. This infection is cosmopolitan in pork-eating populations. The adults are 2 to 7 meters long, and consist of a scolex with four suckers and a rostellar crown of hooklets, a "neck" and somewhat less than 1000 immature to

gravid proglottids. Gravid proglottids, provided with 9 to 13 main lateral uterine arms on each side of the main longitudinal uterine stem usually

worms (*cysticercus* larvæ). Human infection with adult worms is due to ingestion of infected raw pork, infection with the bladder worms is due to swallowing viable eggs of this worm. Adults of this species frequently produce digestive and nervous disorders similar to *T. saginata*; the cysticerci may develop in any tissue of the human body; in the brain they frequently

Diagnosis is based on the demonstration of a characteristic number of uterine arms or of the cysticerci obtained by biopsy or at autopsy.

XX. *Echinococcus granulosus* (hydatid worm).—The adults are typically attached by their scolex to the mucous coat of the small bowel of the dog and its wild relatives; they are minute, only 3 to 6 mm. in length and consist of a scolex, a "neck," one immature, one mature and one gravid proglottid. This infection is cosmopolitan in sheep-raising countries. The gravid proglottid disintegrates in the bowel of the dog, shedding *Tænia*-like eggs which are passed in the dog's feces. Human beings, like sheep, cattle or hogs, which take in food or drink contaminated with these viable eggs, provide opportunity for hatching of the eggs and the migration of their escaping embryos throughout the body, with lodgment in a capillary filter bed, as in the liver, lungs, brain and the shafts of long bones. The embryos develop slowly into many-headed larvæ (hydatid cysts), which sooner or later occasion severe symptoms due to pressure and slow or sudden discharge of their toxic hydatid fluid. An additional hazard is their capacity to implant their daughter heads (scolices) on internal membranes. *Diagnosis* is based in part on the history of a slowly growing mass, especially in the lower right portion of the liver; possibly on demonstration of hydatid "thrill;" but usually preoperatively on specific intradermal, precipitin or complement-fixation tests. (See below, page 690.) At operation or autopsy the cysts usually provide specific diagnostic evidence, including the laminated outer layer and germinative inner layer of the cyst wall with scolices and frequently daughter cysts floating free in the hydatid fluid.

XXI. *Schistosoma japonicum* (Oriental blood fluke).—The adults are typically found in the mesenteric venules draining the small bowel. The males are 12 to 20 mm. long by about 0.5 mm. in diameter, with two acetabula close together at the anterior end and a deep sex canal (a furrow extending from the second [ventral] acetabulum to the posterior extremity); the more delicate females (26 mm. by 0.3 mm.) are usually held in the sex canal of the male. This infection is found in Japan, Formosa, the Philippines, Celebes and in widespread foci in Central, South and West China. Embryonated eggs, laid in the smaller venules, by lytic and mechanical means escape into the perivascular tissues and into the lumen of the small bowel, are passed in feces with blood and mucus, hatch in fresh water, and the escaping larvæ (*miracidia*) attack and enter appropriate species of snails. After a two-fold multiplication within the snail swarms of fork-tailed

SCHISTOSOMIASIS

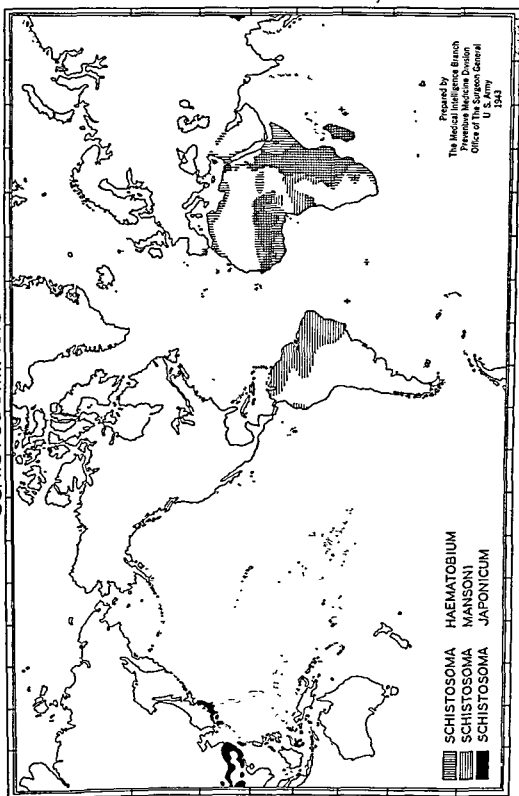


FIG. 80.—Geographical distribution of schistosomiasis.

larvæ emerge and swim about in the water. Human infection results from penetration of larvæ into the skin following swimming or bathing in "infected water." The larvæ migrate through the blood stream via the right heart, the lungs, the left heart, aorta, mesenteric artery, capillaries and venules into the portal stream, then grow and migrate to the venules draining the small bowel. Infection occasions profound toxemia, with fever, anemia, high eosinophilia, neutropenia and monocytosis. Abscesses and pseudotubercles develop around each egg which becomes lodged in the intestinal wall, lymph nodes, liver and other organs, with resultant fibrous thickening of the involved intestine, cirrhosis of the liver with eventual ascites, splenomegaly, emaciation and death. *Diagnosis* is based on the recovery of the characteristic eggs (Fig. 77, U) in the stool, especially in flecks of blood and mucus.

XXII. *Schistosoma mansoni* (Manson's blood fluke).—The adults are minute, thread-like, dioecious parasites. The females are larger than the males and are typically found in the venules draining the urinary bladder or in the pelvic plexuses. They resemble *S. japonicum* and *S. mansoni* but the males are shorter and broader (10 to 15 mm. by 1 mm.). This infection has an extensive distribution throughout Africa, and in the Near East as far as Iraq and Iran. The life cycle of the parasite and the pathogenesis of the disease are similar to *S. japonicum* except that the eggs are passed in flecks of blood, mucus and pus cells at the end of periods of micturition, and that the urinary bladder, pelvic organs and lungs are primarily involved. *Diagnosis* is based on the recovery of the characteristic eggs (Fig. 77, V) in sedimented urine.

XXIII. *Schistosoma haematobium* (Egyptian blood fluke).—The adults are typically found in the venules draining the urinary bladder or in the pelvic plexuses. They resemble *S. japonicum* and *S. mansoni* but the males are shorter and broader (10 to 15 mm. by 1 mm.). This infection has an extensive distribution throughout Africa, and in the Near East as far as Iraq and Iran. The life cycle of the parasite and the pathogenesis of the disease are similar to *S. japonicum* except that the eggs are passed in flecks of blood, mucus and pus cells at the end of periods of micturition, and that the urinary bladder, pelvic organs and lungs are primarily involved. *Diagnosis* is based on the recovery of the characteristic eggs (Fig. 77, X) in sedimented urine.

XXIV. *Fasciola hepatica* (sheep liver fluke).—The adults live in the proximal bile ducts and the gall bladder. They are relatively fleshy and measure 20 to 50 mm. in length by 7 to 13 mm. in breadth. This infection is present throughout the sheep-raising countries of the world and occurs in all types of herbivorous animals, especially sheep and goats. Reported human cases number several hundred. The unembryonated eggs, laid in the bile ducts and gall bladder, pass down the bowel and are evacuated in feces. They require incubation and hatching in fresh water, a two-fold development in certain species of snails, escape of the cercarial larvæ from the snail and encystment on aquatic vegetation, where upon consumption of the vegetation constitutes the source of exposure for man. On ingestion by the definitive host the larvæ excyst, bore through the intestinal wall, traverse the peritoneal cavity and burrow through the liver parenchyma, finally arriving in the bile ducts where they develop to adults. Light infections in man may be essentially symptomless but heavier infections fre-

ment
hyma

cosinophilia may be present. *Diagnosis* is based on the recovery of the characteristic eggs (Fig. 77, P) in feces or in biliary drainage. They are practically indistinguishable from eggs of *Fasciolopsis buski*.

XXV. Fasciolopsis buski (giant intestinal fluke).—The adults are attached by their suckers to the mucous coat of the small bowel, typically at the level of the duodenum. They are fleshy worms measuring 20 to 75 mm.

are evacuated in feces require embryonation in fresh water, hatch, and the miracidial larvæ invade and develop in certain species of snails, with two-fold multiplication. Later the cercarial larvæ emerge from the snails and encyst on certain water plants. When these plants are consumed raw by man, the larvæ excyst, attach themselves to the duodenal mucosa and develop into adults. Infection results in the development of phagedenic lesions at the sites of attachment of the worms to the intestinal mucosa, dysphagia, severe systemic toxemia, with edema and frequently ascites, marked eosinophilia and neutropenia. *Diagnosis* is based on the recovery

ducts. Infection is widely distributed in sheep but is rare in man. Characteristic eggs (Fig. 77, Q) are recovered from feces.

XXVII. Echinostomes.—The adults, which are attached to the mucous coat of the small bowel, typically have a collar of hooklets around the cervical region. These infections are rather common in the Orient. The operculate eggs are smaller but in general resemble those of *Fasciola hepatica* (Fig. 77, P) and are recovered in feces.

XXVIII. Heterophyoid Flukes.—The adults grossly resemble minute mustard seeds and are found attached to, or burrowing into, the mucous coat of the small bowel. Infection is widely distributed in the Far East, in Egypt and in Southeastern Europe. The most prevalent species are *Heterophyes heterophyes* (especially in Egypt) and *Metagonimus yokogawai* (in the Far East). Eggs evacuated in feces are eaten by certain species of snails in which they hatch and develop, with a two-fold multiplication. The cercarial larvæ which emerge from the snails attack and encyst in freshwater fishes. When man eats infected raw fish he acquires the infection. Infection may be essentially symptomless or may produce a mucous diarrhea. At times the eggs infiltrate into the submucous coat of the bowel and are carried in the lymphatics or blood vessels to filter out in heart muscle, producing a type of hypertension simulating wet beriberi; or in the central nervous system, causing symptoms referable to the levels involved. *Diagnosis* is based on the recovery of the characteristic eggs (Fig. 77, R, S) in feces.

XXIX. Opisthorchis felineus (cat . . . transparent, delicate worms measuring . . . breadth. They reside in the distal bile . . . in Eastern and Southeastern Europe, in Russia and Siberia and in parts of French Indo-China. The eggs pass down the bile duct and intestine and

small specimen. Hence a representative sample of the stool can be placed in a 1-ounce metal salve box having a cardboard top used for the patient's name and address or identification number and can be shipped in a mailing

specimen
gical salt
m. square
cover slip. The most serviceable slides for fecal examination measure 75 by 37 to 40 mm. and are alkali-resistant. The film to be examined should be thin enough so that newspaper print can be read through it. The microscope should be equipped with a mechanical stage, preferably built onto the instrument. The film should be examined first with the low-power objective (16 mm.), and the high dry objective (4 mm.) reserved for the actual identification of the object, once it has been found. Rarely is the oil-immersion objective needed for diagnosis of eggs or larvæ present in feces.

When active hookworm or *Strongyloides* larvæ are found in the film, they may be inactivated and stained by drawing a drop of iodine solution through the film. *Enterobius* eggs are not commonly found in feces of infected patients, so that perianal or perineal swabbings are much more satisfactory for the detection of this parasite. *Tænia* eggs may not be found in the stools of persons continually passing ripe proglottids of the worm. A relatively large number of *Ascaris* eggs in an unconcentrated film does not necessarily mean a large number of parent worms, but a comparable number of hookworm or *Trichocephalus* eggs suggests at least a moderate infection. Infertile *Ascaris* eggs are not uncommonly confused with undigested vegetable cells and *Tænia* eggs may be mistaken for stone cells of plants. Plant hairs constitute a common pitfall in searching for *Strongyloides* larvæ.

2. Concentration Technics for the Recovery of Helminth Eggs and Larvæ From Feces.—These consist of floatation and of centrifugalization and their various modifications.

(a) *Floatation Technics* (Willis Levitation Technic).—This consists in mixing a small amount of feces with approximately 10 parts by volume of a saturated solution of unrefined table salt. After about ten minutes most eggs will float to the surface and may be removed with a wire loop to a slide for examination. Concentrated solutions of calcium chloride or of glucose may be substituted for the solution of table salt but are less economical. Eggs of *Schistosoma* spp. are usually shrunken in these saline concentrates, so as to make identification difficult, while eggs of *Clonorchis sinensis* and heterophoid flukes are too heavy to rise to the surface. Floatation technics are not satisfactory for helminth larvæ. Usually considerable débris from the feces also rises to the top of the feces-saline mixture and obscures the eggs. This difficulty may be obviated by straining the mixture through a layer of cheesecloth before allowing floatation to occur. Floatation is the simplest concentration technic and can be utilized in the field or in laboratory. The equipment is very meager.

also allows concentration of protozoan cysts which may be in the specimen. A 2- to 5-gm. fecal specimen is thoroughly mixed with about 10 parts by

volume of tap water, and is then strained through a single layer of 16- to 20-mesh cheesecloth (to remove debris) into a 13 by 100-mm. serological pyrex glass tube. Because of their rounded bottoms these tubes are better for fecal concentrates than the conventional 15-cc. centrifuge tubes, since the material does not easily pack in the base of the tube. The tubes are revolved in the centrifuge at a moderate speed for thirty to sixty seconds, are then removed, the supernatant liquid poured off, the tubes filled with water, in which the fecal material is thoroughly mixed, and are again centrifugalized. Theoretically, the centrifugalization should be repeated until the supernatant fluid is clear, but in practice two spinnings are usually satisfactory.

(c) *The Clayton-Lane Technic.*—This is a refined combination of floatation and centrifugalization, whereby the quantity of the eggs in a weighed sample of feces may be accurately estimated. The method is of particular value for research workers but requires special apparatus and is not practical for the average clinical laboratory.

(d) *The Zinc Sulfate Centrifugal Floatation Technic.*—The steps in this technic are as follows:

(1.) A fecal suspension is prepared by comminuting about 10 parts of lukewarm tap water with one part of the stool specimen (about the size of a pecan).

(2.) Approximately 10 cc. of the suspension are strained through one layer of *wet* cheesecloth (in a small funnel) into a Wassermann tube.

(3.) The preparation in the tube is then centrifugalized for forty-five to sixty seconds at top speed of an International clinical centrifuge (ca. 2500 r.p.m.). The supernatant fluid is poured off, 2 or 3 cc. of water are added, the sediment is broken up by shaking or tapping, and additional water is added to fill the tube.

(4.) Repeat "3" (usually 3 or 4 times) until the supernatant fluid is clear.

(5.) The last supernatant fluid is poured off, 3 to 4 cc. of zinc sulfate solution of the specific gravity 1.180 (33 per cent solution) are added, the packed sediment is broken up and enough zinc sulfate solution is added to fill the tube to about one-half inch of the rim.

(6.) The tube is centrifugalized for forty-five to sixty seconds at top speed.

(7.) Several loopfuls of diagnostic material floating in the surface film are removed by means of a bacteriological loop onto a clean slide, 1 drop of D'Antoni's iodine stain is added and the preparation agitated manually

for the concentration at one time of protozoan cysts, helminth eggs and larvae in a diagnosable and viable state.

✓(e) *Simplified Zinc Sulfate Centrifugal Floatation Technics.*—Otto, Hewitt and Strahan (1941) have utilized a zinc sulfate solution of specific gravity 1.180 as a direct floatation medium, without screening the comminuted feces through cheesecloth. The technic is performed in shell vials (5 cm. high by 1.8 cm. diameter), and the harvest of protozoan cysts and helminth eggs is obtained on superimposed $\frac{1}{4}$ -inch square cover glasses. This method was found to be slightly inferior to the original technic of Faust and his

associates (1938, 1939) for protozoan cysts but appreciably better for helminth eggs.

Summers (1942) has recommended a centrifugal floatation technic, utilizing zinc sulfate solution of specific gravity 1.200, without straining, for formalinized feces. This is apparently fairly satisfactory for helminth eggs but does not allow discriminate differentiation of protozoan cysts.

(f) *Sedimentation*.—Eggs of *Schistosoma mansoni* and *S. japonicum* are best concentrated by diluting a generous portion of the suspected fecal specimen in water, pouring it into a large urinalysis glass and then allowing sedimentation to occur. Several decantations and additional washings are at times necessary to provide adequate concentration. The eggs may then be pipeted from the bottom sediment. Sedimentation should be completed within four to six hours after dilution has been made, else hatching may occur.

3. *The Stoll Technic*.—This method is used in the quantitative estimation of eggs in a weighed sample of feces. It is not a concentration but a dilution method. The technic is as follows: 4 gm. of feces are weighed out and placed in a graduate, small Erlenmeyer flask, or large test tube having a mark indicating a 60-cc. level, and decinormal sodium hydroxide is added up to the 60-cc. mark. Several small glass beads are now added, the container closed with a rubber stopper and the contents shaken until the feces are thoroughly comminuted. A hard fecal specimen should be left in the liquid overnight to secure adequate disintegration. When proper comminution has been obtained, the mixture is thoroughly shaken up and 0.15 cc. of the suspension is drawn up into a capillary pipet, discharged

of eggs per gm. of feces. The estimated daily output of eggs can then be secured by multiplying the number per gm. by the total weight of a twenty-four-hour fecal specimen. The estimate obtained is usually accurate within 10 to 20 per cent, but varies per gm. of feces for a particular species of helminth, depending on the consistency of the feces (whether liquid, semi-formed, formed or hard). It also varies in total daily output per female worm in a given period of time, depending on the number of worms in the infection, as well as on the duration of the infection.

4. *Worms or Portions of Worms Migrating Out of the Anus*.—Adult *Enterobius* females habitually migrate out of the anus and their eggs commonly are shed into the perianal and perineal skin folds. The worms may be passed in the feces but more commonly crawl out of the anus at night. Patients who believe themselves to be suffering from infection with seatworms should collect one or more specimens of the worms, preserve them in rubbing alcohol and bring them to the clinical laboratory for verification. Mature and immature *Ascaris* are frequently passed spontaneously by the host. Tapeworm proglottids (species of *Tenia* or *Dipylidium caninum*) singly or in chains, are usually discharged periodically in the stools of infected patients. Specific identification of these worms can most readily be made from the unpreserved moist proglottids.

5. *Anal Swab Technics*.—The most efficient method is the National Institute of Health (NIH) swab technic (Hall, 1937; Sawitz, Odom, and Lincicome, 1939).

The NIH Swab Technic.—The NIH swab, a glass rod tipped with cellophane held in place with a rubber band, is employed to swab the perianal area for *Enterobius* eggs. The cellophane with the adhering material is removed from the rod, is flattened between two glass slides and examined with low power of the microscope. While a single swab examination provides a high percentage of the total positives, not less than seven swab examinations should be made before a suspected patient is diagnosed as negative. This technic provides many times more positives than the most efficient technics for concentration of eggs from feces (i. e., zinc sulfate centrifugal floatation, brine floatation, etc.).

Perianal swabbing is also recommended for the recovery of eggs of *Tænia* and of *Schistosoma mansoni*.

II. Examination of Urine.—Urine is the almost exclusive body excretion in which eggs of *Schistosoma hæmatobium* are discharged. The specimen of urine is collected in a urinalysis or sedimentation glass and the eggs allowed to sink to the bottom, together with erythrocytes and pus cells. The bottom sediment is then pipeted onto a fecal slide and examined microscopically. In Bancroft's filaria infection with chyluria the microfilariae are discharged in the urine. They may readily be recovered from centrifugalized specimens.

III. Examination of Sputum.—Eggs of *Paragonimus westermani* are commonly coughed up and discharged in the sputum. In *Paragonimus* patients with pulmonary involvement, the sputum may or may not be stained with blood, but it is invariably sprinkled with minute iron-brown flecks, which are the eggs of the parasite. In persons infected with *Strongyloides*, less commonly with hookworms and *Ascaris*, during the passage of the migrating larvæ from the bronchioles up to the epiglottis, the larvæ may be recovered from the sputum. Likewise, patients having *Syngamus* worms in the trachea will at times discharge eggs or the paired adult worms after paroxysms of coughing.

IV. Recovery of Larvæ and Microfilariae From the Blood.—During the

Microfilariae are most usually found in thick blood films. In *Wuchereria bancrofti* and *W. malayi* infections they are most readily demonstrated in blood taken between 10 P.M. and 4 A.M.; in *Loa loa* infection they are most numerous in the peripheral circulation around mid-day. *Onchocerca volvulus* larvæ rarely, if ever, circulate in the blood stream.

A convenient way of preparing the thick film is to draw up 20 cmm. of blood at the appropriate time into a blood-counting pipet and discharge it immediately upon an absolutely clean microscopic slide in a spiral the

in a drying oven at 35° C.), and is then immersed vertically in dilute Giemsa's stain, or in Wright's stain diluted 1 to 30 with distilled water. After thirty to sixty minutes, during which both dehemoglobinization and staining occur, the slide is removed from the solution without washing, the excess solution drawn off with filter paper and the slide dried without

blotting. As an alternative, the dried thick film may be dehemoglobinized in dilute hydrochloric acid (0.5 per cent), neutralized in lithium carbonate solution (1 per cent) and then stained with iron-alum hematoxylin.

V. Biopsied Material and That Obtained at Operation.—Biopsy technic is useful in recovery of suspected organisms which may be present in the skin, the subcutaneous tissues and somatic musculature. In patients with trichinosis (after the fourth week), small strips of deltoid muscle are removed under local anesthesia and are digested with pepsin by the Bachman technic. The larvæ should be searched for in the sediment after centrifugation. Larvæ of *Tænia solium* (*Cysticercus cellulosæ*) are at times present in the skin, subcutaneous tissues or superficial muscles, where they appear as soft, whitish, elevated tumors, which usually produce no pain. After removal from the patient, the suspected tissue may be fixed and sectioned *in toto* or the cysticercus may be dissected out of its adventitious capsule, the bladder opened by puncture and the specimen mounted on a slide under a cover glass and examined under a microscope. In geographical areas where *Onchocerca volvulus* is present, indurated nodules appear at various sites on the patient's body, particularly on the head and at the junction of the long bones. Upon removal of the suspected tissue it may be fixed and sectioned for examination. In patients operated on for infection with hydatid cyst (*Echinococcus granulosus* larvæ) it is usually imperative for the surgeon to have immediate information regarding the character of the fluid aspirated from the cystic cavity (*i. e.*, whether it contains viable scolices and daughter cysts, whether it is sterile or whether it is secondarily infected). The clinical pathologist should have his microscope nearby the operating amphitheater and be prepared to provide immediate diagnosis of the material while the patient is on the operating table.

VI. Helminths Obtained Following Chemotherapeusis.—This applies exclusively to parasites of the intestinal tract. In the case of tapeworm infections it is important to examine all stools for at least twenty-four hours (preferably forty-eight hours) following anthelmintic medication, in order to determine the efficiency of the treatment. Due to post-treatment purgation, the stools are usually liquid and the worms are readily recognized and isolated. Examination is facilitated by placing small amounts of the evacuated material in a large flat Petri dish. Special care must be used to find and identify the heads of tapeworms, since cure of tapeworm infection can be guaranteed only in case the heads are dislodged from the bowel wall. In nematode infections, the adult worms may be similarly recovered, but the usual procedure is to allow several days to elapse after chemotherapeusis, and then examine the feces of the patient for eggs or larvæ of the residual worms. In strongyloidiasis and in clonorchiasis, follow-up examinations must be repeated several times in order to determine the value of the therapeusis.

VII. Helminths Obtained at Autopsy.—Most satisfactory results are always secured from autopsies performed within two to four hours after death. After the gastro-intestinal tract has been removed from the body it should be opened along the line of lesser curvatures and the contents from the several levels collected separately for subsequent examinations. Hookworms and whipworms should be removed by gentle traction from their attachment to the bowel wall. *Strongyloides* in the wall

may be obtained by careful scraping of the mucosa, particularly of the duodenum and jejunum, and examining in a 2 per cent Glauber salts solution to dissolve the mucus, but blocks of supposedly infected tissue should always be cut out and fixed as soon as possible in Zenker's fluid, later sectioned and examined microscopically. Minute worms, as *Trichinella* adults and heterophyoid flukes, are most readily obtained by placing the opened bowel in luke-warm physiological salt solution, shaking vigorously and examining the liquid for the worms either in a large flat Petri dish or by passing the liquid through fine bronze screen or bolting cloth. For *Clonorchis*, *Opisthorchis*, *Fasciola* and *Dicrocoelium* infections, the gall bladder and biliary passages must be opened and carefully examined. For *Schistosoma mansoni* and *S. japonicum* infections, the liver should be removed intact with its connections to the entire intestinal tract, and the mesenteric venules carefully examined. If the blood has not yet clotted, the venules may be irrigated by forcing a citrate solution into the main mesenteric arteries and allowing it to flow out through openings made in the main extrahepatic portal veins. If clotting has already occurred, the veins must be carefully dissected to recover the worms. Likewise, the intrahepatic portal vein must be opened and search for the worms made there. Representative blocks of intestine, mesenteric lymph nodes and liver should always be fixed and sectioned. For *S. hæmatobium*, the urinary bladder and other organs of the pelvic region, together with their blood supply, should be removed and similarly examined. *Paragonimus* is most commonly found within cystic tumors in the depth of the lungs but the worms may have developed in the abdominal viscera or even in the brain. *Echinococcus* cysts most frequently occur in the liver but have been recovered from practically every organ and tissue of the body. The adult Bancroft's filaria worms may develop in lymph nodes and lymphoid tissue in any portion of the body, but are most commonly located in the groin, medially above the testes or at the proximal end of obstructed lymph tracts.

Most helminths do not require delicate technics for satisfactory preservation. Tapeworms and trematodes usually fix well in steaming (not boiling) 2 per cent formaldehyde (5 per cent formalin), and nematodes may be fixed in the same medium to which glycerol (1 part to 10 by volume) has been added before the fixative is heated. In case large tapeworms are desired for demonstration specimens, they should be allowed to die and should be placed in the desired position before fixation. Sections of tissues containing helminths and their eggs or larvæ should be fixed in Zenker's fluid. For museum specimens, in which it is desirable to retain the natural color of tissues, the Kaiserling technic should be employed. For staining microscopic sections containing helminths, Delafield's hematoxylin with eosin is fairly satisfactory. For the more delicate fixation of helminths, needed for study of the more detailed histology and cytology of the worms Gilson's or Bouin's fluid is recommended.

Eggs of helminths obtained from centrifugalization or sedimentation of feces or urine are satisfactorily fixed and preserved in steaming 4 per cent formaldehyde. *Paragonimus* eggs in sputum may be preserved in a phenol-glycerol solution (phenol, 1 per cent; glycerol, 5 per cent; distilled water, 94 per cent).

VIII. Serological Tests for Helminthic Infections.—Only the most important practical tests can be listed here. These include: (1) the intradermal test; (2) the precipitin reaction and the complement-fixation test.

1. The Intradermal Test.—(a) *Trichinosis*.—The intradermal test is a valuable aid for diagnosing infection with *Trichinella spiralis*. It is particularly helpful in mild cases, which manifest only vague symptoms. The following is an adaptation of the Bachman technic (1928). Antigen is prepared from laboratory rats infected with *Trichinella*. For each 80 gm. of meat from the sacrificed rat 1500 cc. of a 0.6 per cent pepsin—0.3 per cent HCl solution is used to digest the larvae out of the meat, the material being kept at 37° C. for five to twelve hours and shaken from time to time. The digest is then poured through six layers of cheesecloth, diluted with an equal amount of water and allowed to stand in a graduate for two hours. The upper third of the liquid is drawn off and replaced with warm tap water. This process is repeated six or eight times until the supernatant fluid is clear. The purified material is left in a sedimentation glass overnight and next morning is placed in a Petri dish, allowed to dry and then transferred to a beaker with ether to remove lipoids. After twenty-four hours the ether is removed from the top and the residue dried *in vacuo* over sulfuric acid for forty-eight hours. The dry yield is pulverized in a clean dry mortar and kept in sterile ampules or dissolved in Coca's or McCoy's solution, 1 to 100 parts by weight. This latter constitutes the stock solution. For intradermal tests it is diluted 1 to 50 to secure a 1 to 5000 dilution. This is kept on ice until used. In the test, 0.1 cc. of antigen is introduced intracutaneously on one forearm and an equal amount of the solution lacking the antigen is injected intracutaneously on the other forearm. In positive cases (whether clinical or sub-clinical in type) a small white swelling appears immediately around the injected site, surrounded by an unraised irregular erythematous area of about 5 cm. diameter. The reaction reaches its maximum in about ten minutes and begins to fade in fifteen to twenty minutes. In negative cases there is no reaction. It is always desirable to supplement the intradermal test with a precipitin test (*vide infra*), especially to determine if the infection has been of recent origin.

(b) *Hydatid Cyst*.—The antigen usually employed consists of sterile hydatid fluid obtained by puncture of unilocular hydatid cysts of sheep, pigs, oxen or human cases. Only clear fluid from active cases is used. Preferably several samples are pooled to obtain uniform reactions. The liquid antigen is filtered, incubated to test its sterility, and placed in sealed ampules on ice. The most potent hydatid antigen is that provided by the Dennis technic (1937).

Freshly aspirated, bacteriologically sterile, hydatid fluid from cysts of the liver and lungs of infected cattle and sheep constitutes the source of the antigen. About 1 liter of the fluid is chilled, acidified by the addition of 5 per cent trichloroacetic acid, and placed in the ice-box overnight to accelerate flocculation. The precipitate is obtained by repeated centrifugalization and is next washed in distilled water to remove excess acid. It is then suspended in about 50 cc. of distilled water and 10 per cent sodium hydroxide added, drop by drop, until practically all of the protein is in solution. The insoluble residue is collected by centrifugalization and discarded. The solution is then chilled, the protein reprecipitated by the addition of 1 N glacial acetic acid and left in the ice-box

overnight. It is then recentrifugalized, washed free of acid and evaporated in a drying oven at 37° C. or over calcium chloride. The dry precipitate is ground in a mortar and stored over calcium chloride in a desiccator. About 100 mg. of purified antigen may be obtained from each liter of hydatid fluid. Stock antigen solution is made up 1 to 1000 in slightly alkaline physiological salt solution, sterilized by filtration through a fine sintered glass filter, and stored over calcium chloride in a desiccator. This solution is used for intradermal tests.

For intradermal tests the purified powdered Dennis antigen is diluted 1 to 10,000 in physiological salt solution, and 0.2 cc. of this solution employed. Possibly the most specific skin test may be obtained by using the polysaccharide portion of antigen extracted from hydatid scolices.

2. The Precipitin Reaction.—This constitutes a delicate specific test but is subject to misinterpretation on the part of unskilled workers. It is particularly valuable in checking diagnoses made by intradermal tests, in patients suspected of harboring *Trichinella* larvae or hydatid cyst. Eight serological tubes are arranged in series. Into the first six, 0.2 cc. amounts of patient's serum are introduced. In the seventh tube an equal amount of normal human serum is placed and into the eighth an equal amount of infected rabbit's serum is placed. The tubes are respectively overlaid with the following solution: (1) 0.2 cc. *Trichinella* (or hydatid) antigen, 1 to 100 in Coca's solution; (2) 0.2 cc. antigen, 1 to 200; (3) 0.2 cc. antigen, 1 to 400; (4) 0.2 cc. antigen, 1 to 800; (5) 0.2 cc. antigen, 1 to 1600; (6) 0.2 cc. Coca's solution lacking the antigen; (7) 0.2 cc. antigen, 1 to 100. (8) 0.2 cc. antigen, 1 to 100. Negative sera remain perfectly clear, while positive sera develop a white ring, within thirty minutes, at the level of contact with the antigen, and the antigen usually becomes cloudy. This technic detects positive cases without a clinical history of trichinosis as well as clinically positive ones, but is less sensitive in old chronic cases than is the intradermal reaction (Hall, 1937).

3. The Complement-fixation Test.—This serological test has been employed in the diagnosis of hydatid cyst, schistosomiasis, trichinosis, etc. In general, it resembles the Wassermann technic for syphilis.

(a) *Hydatid Cyst.*—The antigen consists of hydatid fluid removed aseptically from known infected human cases or from infected domestic mammals. Contaminated antigen must not be used. Except in heavily endemic areas antigen is difficult to obtain. For this test the Dennis purified powdered antigen (see above) is diluted to 1 to 5000 concentration and is utilized as in the Kolmer modification of the Wassermann test. This antigen is sensitive, specific, not anti-complementary and apparently gives no false positive tests.

(b) *Schistosomiasis.*—The antigen is group-specific. It is usually prepared as an alcoholic extract of molluscan tissues infected with mammalian schistosome cercariae (1 cc. 95 to 96 per cent alcohol for each infected snail liver). After extraction at 37° C. for twenty-four hours, it is filtered, the filtrate then evaporated and thoroughly dried, weighed and dissolved in physiological salt solution (1 to 40 by weight). Most authorities agree that the antigen is a lipid rather than a protein. This test is most useful in patients passing through the incubation period of the disease, before eggs are being discharged and in old chronic cases, in which extensive fibrosis

of the bowel or bladder wall usually prevents evacuation of eggs. Positive cases should be checked with a routine Wassermann test to preclude syphilis.

DIAGNOSTIC KEY FOR THE IDENTIFICATION OF THE MORE COMMON HELMINTH EGGS AND LARVÆ*

- 1 (19, 24). Eggs. 2
- 2 (14). *Provided with an operculum*. 3
- 3 (9). *Unembryonated*. 4
- 4 (5). *In sputum*. Broadly ovoidal, dark golden-brown; moderately thick-shelled, with relatively flat but distinct operculum and thickened abopercular end; size: 80-118 x 48 x 60 μ
Paragonimus westermani (Fig. 77, V).
- 5 (4). *In feces* 6
- 6 (7, 8). Large, hen's-egg-shaped, light yellowish or greenish-brown, with relatively thin shell and small, indistinct operculum.
i. Size: 130-150 x 63-90 μ
Fasciola hepatica and *Fasciolopsis buski* (Fig. 77, P).
ii. Size: 83-116 x 58-69 μ
Echinostoma ilocanum.
iii. Size: 120-130 x 80-90 μ
Echinostoma malayanum.
- 7 (6, 8). Long, narrowly ovoidal to elliptical, with a small, distinctly domed operculum; size 150-170 x 60-70 μ
Gastrodiscoides hominis (Fig. 77, W).
- 8 (6, 7). *i.* Broadly barrel-shaped, relatively thick-shelled, with a broad, slightly domed operculum; size: ca. 70 x 45 μ
Diphyllbothrium latum (Fig. 77, M).
ii. Narrowly barrel-shaped, relatively thick-shelled, with a narrow, distinctly domed operculum; size: ca. 60 x 35 μ
Diphyllbothrium houghtoni, *D. mansonii*, *D. erinacei*, *D. decipiens*, etc. (Fig. 77, N).
Likewise *Paragonimus westermani* in sputum eggs may be swallowed and passed in the feces. See "4" above.
- 9 (3). *Fully embryonated* 10
- 10 (11). Medium-sized (38-45 x 22-30 μ), with a thick, dark-brown shell, having a distinctly domed operculum.
Dicrocoelium dendriticum (Fig. 77, Q).
- 11 (10). Minute eggs, with a distinct operculum. 12
- 12 (13). With enclosed miracidium having asymmetrically arranged lytic glands.
i. Size: ca. 30 x 11 μ
Opisthorchis felinus (Fig. 77, T).
ii. Size: 27.3-35.1 x 11.7-19.5 μ
Clonorchis sinensis (Fig. 77, U).
- 13 (12). With enclosed miracidium having bilateral symmetry of lytic glands.
i. Size: 28-30 x 15-17 μ
Heterophyes heterophyes (Fig. 77, R).
ii. Size: 26.5-28 x 15.5-17 μ
Metagonimus yokogawai (Fig. 77, S).

* From Faust's Human Helminthology, pp. 586-590, 1939.

- 14 (2). *Lacking an operculum.* 15
- 15 (16, 17, 18). *Fully embryonated, containing a rhabditoid larva; egg medium-sized (50-60 x 20-30 μ); narrowly ovoidal, relatively thick-shelled, flattened on one side.*
Enterobius vermicularis (Fig. 77, C).
- 16 (15, 17, 18). *Fully embryonated, containing a ciliated larva; with a light yellowish-brown shell having a spine.*
 i. *Narrowly ovoidal, with a distinct terminal shell spine; size: 112-170 x 40-70 μ .* *Schistosoma hæmatobium* (Fig. 77, X).
 ii. *Narrowly ovoidal, with a distinct lateral shell spine; size: 114-175 x 45-68 μ .* *Schistosoma mansoni* (Fig. 77, Y).
 iii. *Broadly ovoidal, with an inconspicuous, small, hooked spine; size: 70-100 x 50-65 μ .* *Schistosoma japonicum* (Fig. 77, Z).
- 17 (15, 16, 18). *Fully embryonated, containing a non-ciliated embryo (onchosphere) possessing 3 pairs of hooklets.*
 i. *With a thick, brown, radially-channelled, outer shell; subspherical; size: 31 to 43 μ in diameter.*
Tænia saginata and *Tænia solium* (Fig. 77, J).
 ii. *With a thin, hyaline, outer shell; polar thickenings with filaments on inner shell; spherical to subspherical; size: 30-47 μ in diameter.* *Hymenolepis nana* (Fig. 77, K).
 iii. *With a moderately thin, light yellowish-brown, outer shell, polar thickenings without filaments on inner shell; subspherical, size: 60-79 x 72-86 μ .* *Hymenolepis diminuta* (Fig. 77, L).
 iv. *With thin, hyaline, outer shell; spherical; size: 25 to 49 μ in diameter; several eggs typically enclosed in a mother embryonic membrane.* *Dipylidium caninum* (Fig. 77, O).
- 18 (15, 16, 17). *Unembryonated or incompletely embryonated.*
 i. *Shell narrowly barrel-shaped, dark brown, with a plug-like, semi-opaque, whitish swelling at each end; size: 50-54 x 22-23 μ* *Trichocephalus trichiurus* (synonym: *Trichuris trichiura*) (Fig. 77, I).
 ii. *Shell usually provided with an outer, mammillated, albuminoid cover, characteristically bile-stained; with thick, hyaline, outer shell; fertile eggs broadly ovoidal; size: 45-75 x 35-50 μ ; infertile eggs irregularly elongated-ovoidal; size: 88-93.5 x 38.5-44 μ* *Ascaris lumbricoides* (Fig. 77, A, B).
 iii. *Shell thin, hyaline, elongated-ovoidal, with narrowly rounded ends; typically with morula-stage embryo; size: 73-80 x 40-46 μ* *Trichostrongylus colubriformis* or *T. probolurus*; 84-90 x 46-50 μ *T. ritrinus*; 75-91 x 39-47 μ *T. orientalis* (Fig. 77, H).
 iv. *Shell thin, hyaline, ovoidal, with bluntly rounded ends; size: ca. 60 x 40 μ .* *Ancylostoma duodenale* or *A. braziliense*; 64-76 x 36-40 μ *Necator americanus* (Fig. 77, D, E); 50-58 x 30-34 μ parasitic generation of *Strongyloides stercoralis* (rarely found unhatched in feces).
- 19 (1, 24). *LARVÆ* 20
- 20 (21, 22, 23). *Moderately short, with muscular esophagus.*
 i. *Esophagus having only a posterior bulbar swelling; pre-esophageal chamber very short.*
Strongyloides stercoralis (rhabditoid larva) (Fig. 77, G).

- ii. Esophagus having only a posterior bulbar swelling; pre-esophageal chamber long and narrow.
Ancylostoma or *Necator* (rhabditoid larva) (Fig. 77, F).
- iii. Esophagus having both a median and a posterior bulbar swelling
 Most species of *Rhabditis* (Fig. 79).
- 21 (20, 22, 23). With long, attenuate, caudal extension and with muscular esophagus.
 - i. Esophagus having both a median and a posterior bulbar distention.
 Some species of *Rhabditis*.
 - ii. Esophagus having only a slight posterior bulbar swelling; with distinctly striated cuticula and a pair of minute pockets on either side of the anus; size: 500-750 x 15-25 μ
Dracunculus medinensis
 (discharged by mother worm
 from cutaneous lesion into
 water). (Fig. 85, p. 676).
- 22 (20, 21, 23). Elongate, narrow, with long, narrow, muscular esophagus.
 - i. With minute forking at caudal extremity.
Strongyloides stercoralis
 ("filariform" larva). (Fig. 78, B).
 - ii. With sharply pointed caudal extremity.
Ancylostoma or *Necator*
 ("filariform" larva). (Fig. 78, A).
- 23 (20, 21, 22). Elongate, narrow, characteristically coiled, with non-muscular esophagus *Trichinella spiralis*
 (rarely recovered in feces or blood). (Figs. 80, 81).
- 24 (1, 19). MICROFILARIE. 25
- 25 (26). *Provided with a sheath.*
 - i. Without nuclei in tip of tail; in circulating blood, in most endemic areas exhibiting strict nocturnal periodicity; size: 244-296 x 8 μ *Microfilaria bancrofti* (Fig. 83, A).
 - ii. With two distinct nuclei at tip of tail; in circulating blood, exhibiting partial nocturnal periodicity; size: 177-230 x 3.4-3.8 μ *Microfilaria malayi* (Fig. 83, B).
 - iii. With nuclei extending into tip of tail; in circulating blood, exhibiting partial diurnal periodicity; size: 250-300 x 6-8.5 μ *Microfilaria loa* (Fig. 83, C).
- 26 (25). *Without a sheath.*
 - i. With nuclei extending into tip of tail; in circulating blood, exhibiting slight nocturnal periodicity; size: 160-200 x 4.5-6 μ
 - ii.
 - iii.

Microfilaria rostratus (Fig. 83, F).

A SUMMARY OF IMPORTANT (OR COMMON) HELMINTHIC DISEASES OF MAN

Diagnostic medium	Stage encountered	Common name of etiologic agent	Scientific name of etiologic agent	Habitat of etiologic agent in man
Feces	Egg	Whipworm	<i>Trichocephalus trichiurus</i> (<i>Trichuris trichiura</i>)	Cecum, appendix, colon, posterior segment of ileum (attached)
Feces	Egg	Hookworm	<i>Ancylostoma duodenale</i> <i>A. braziliense</i> (in the Tropics) <i>Necator americanus</i>	Middle third of small intestine (attached) idem. idem.
Feces	Egg Adult	Pinworm Seatworm Oxyuria	<i>Enterobius vermicularis</i> (<i>Oxyuris vermicularis</i>)	Appendix, rectum and lower bowel, perianal region (attached or free)
Feces	Egg	Giant intestinal roundworm	<i>Ascaris lumbricoides</i>	Lumen of small intestine (free)
Feces	Itchabittoid larva (less commonly filariform larva)	Threadworm	<i>Strongyloides stercoralis</i>	Mucosa of small intestine, especially duodenum (embedded)
Feces	Egg	Manson's blood fluke	<i>Schistosoma mansoni</i>	Venules of mesenteric vessels draining large bowel; portal vessel
Feces	Egg	Oriental blood fluke	<i>Schistosoma japonicum</i>	Venules of mesenteric vessels draining small bowel; portal vessel
Feces	Egg	Giant intestinal fluke	<i>Fasciolopsis buski</i>	Duodenal or jejunal wall (usually attached)
Feces	Egg	Chinese liver fluke	<i>Clonorchis sinensis</i>	Distal bile passages (attached to wall)
Feces	Egg	Broad fish tapeworm of man	<i>Diphyllobothrium latum</i>	Lumen of small intestine (attached by "head")
Feces	Egg	Dwarf tapeworm	<i>Hymenolepis nana</i>	Lumen of small intestine (attached by "head")
Feces	Egg, more commonly proglottid	Pork tapeworm	<i>Taenia solium</i>	Lumen of small intestine (attached by "head")
Feces	Egg, more commonly proglottid	Beef tapeworm	<i>Taenia saginata</i>	Lumen of small intestine (attached by "head")
Peripheral blood	Microfilaria	Bancroft's filaria (sheathed embryo), Malaysian filaria (sheathed embryo)	<i>Wuchereria bancrofti</i> <i>W. malays</i>	Lymphoid tissues and lymphatic vessels
Peripheral blood	Microfilaria	Loa worm, eye worm	<i>Loa loa</i>	Subcutaneous tissues (migrating)
Peripheral blood	Microfilaria	Persistent filaria	<i>Acanthocheilonema perlati</i>	Mesenteric, pleural, pericardial cavities
Urine	Egg	Vesical blood fluke urinary "bilharzia"	<i>Schistosoma haematobium</i>	Vesical and pelvic plexuses, portal system, pulmonary arterioles
Sputum (also 40% in feces)	Egg	Oriental lung fluke	<i>Paragonimus westermani</i>	Lungs, bronchioles and smaller air passages (within adventitious capsules)
Tissue (a) skin	Larva	"Larva migrans"	<i>Ancylostoma</i> spp., especially <i>A. braziliense</i>	Stratum germinativum (migrating)
(b) Skeletal muscles	Larva	Trichina worm	<i>Trichinella spiralis</i>	Larva encysted in muscle, adults in intestinal wall
(c) Tumors in connective tissues	Adult Microfilaria	Convolutad filaria, producing costal erysipelas, "blinding" filaria	<i>Onchocerca volvulus</i>	Tumors in subcutaneous connective tissue (embedded)
(d) Papule → cutaneous lesions	Adult	Dragon worm Guinea worm Medina worm	<i>Dracunculus medinensis</i>	Subcutaneous tissue (migrating)
(e) Subcutaneous tissues; papules	Sparganium larva	Sparganium	Sparganium spp., especially <i>S. mansoni</i>	Subcutaneous tissues and muscle fascia (sedentary or migrating)
Exploratory		H3 dated cyst	<i>Echinococcus granulosus</i>	Liver, lungs, kidneys, spleen, intestinal wall, heart, brain, etc (encapsulated)

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PART VIII

Entomology

CHAPTER XXXVIII

INSECTS OF MEDICAL IMPORTANCE

By WILLIAM B. HERMS

ACCURATE identification of species is fundamental to the application of successful measures employed in the control of insects of medical importance. These measures must fit the particular offending species of insect, tick or mite. For purposes of identification a compound microscope is usually required, although a hand lens, preferably 10X, will suffice in many instances. In order to use identification keys, knowledge of the external morphology of insects and their allies is necessary. While more detailed keys will be required for technical purposes, the brief keys in this chapter are adequate for practical purposes.

INSECTS

General Characteristics.—The most striking characteristic of adult insects is the separation of the body into three divisions—the head, the thorax and the abdomen (Fig. 87). The head bears a pair of antennæ, the mouth parts, and eyes when present. The thorax is divided into pro-, meso-, and metathorax, each of which bears ventrad a pair of legs (three pairs altogether). Normally the meso- and metathorax each bear dorsally a pair of wings. In the *Diptera* there is, however, only one pair of wings and these are on the dorsum of the mesothorax; the metathorax bears a pair of knobbed organs known as halteres (Fig. 87). In the *Anoplura* (sucking lice) and a few other orders, such as the *Siphonaptera* (fleas), the wings are absent. The abdomen of all insects is devoid of appendages except the terminalia. These, particularly in the males, are significant in the classification of mosquitoes (*Culicidæ*).

To determine the Order to which an insect belongs (as many as 30 Orders are recognized in the *Insecta*) one ordinarily uses only the character and structure of the wings, if present, and the type of mouth parts. When functional wings are present it will be observed that the veins, rib-like structures, divide the wings, which may be wholly membranous or more or less leathery, into areas called cells (Fig. 88, *B*). The mouth parts are either mandibulate, *i. e.*, biting, as in cockroaches, or haustellate, *i. e.*, sucking, often piercing, as in the bugs, certain flies, and mosquitoes.

Insect Metamorphosis.—In order to attain the size and development of the parents, the young insect undergoes greater or less change in size, form and structure. This series of changes is termed metamorphosis. Increase in size is achieved by a series of molts. The least change is found in such forms as the so-called "silver fish moth" (*Lepisma saccharina*)

greater size and wingedness with each molt until maturity is reached. The greatest difference between the newly hatched young and the parents occurs in such forms as the housefly, flea (Fig. 90), and mosquito (Fig. 91). In these forms the newly hatched insect has no outward resemblance to

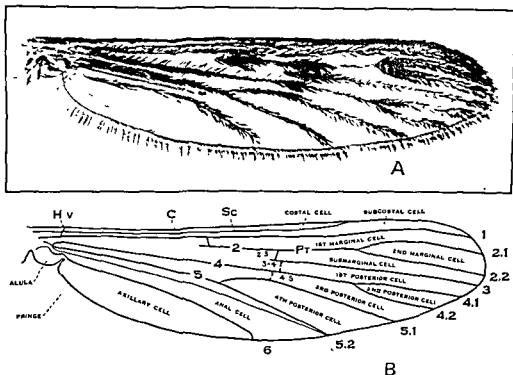


FIG. 88.—A, Wing of *Anopheles*. B, Diagram of wing of *Anopheles* mosquito showing venation. H-v, humeral cross vein; C, costal vein, Sc, subcostal (auxiliary) vein.

TABLE 87.—KEY TO THE ORDERS OF INSECTS (ADULT) OF MEDICAL IMPORTANCE

- | | |
|---|--|
| 1. Wingless | 2 |
| Winged | 4 |
| 2. Mouth parts, mandibulate. | Body compressed dorso-ventrally |
| | <i>Mallophaga</i> (biting lice of birds and mammals) |
| Mouth parts, suctorial | 3 |
| 3. Mouth parts, minute and retracted. | Body compressed dorso-ventrally. |
| | <i>Anoplura</i> (sucking lice) |
| Mouth parts, conspicuous. | Body compressed laterally, with many spines, leathery body, legs adapted for jumping, hind pair of legs larger than others |
| | <i>Siphonaptera</i> (fleas) |
| 4. Body not compressed. | Usually one pair of membranous wings, one pair of halteres (small knobbed processes on metathorax). |
| | <i>Diptera</i> (flies) |
| 5. | 5 |
| dibulate | <i>Colcoptera</i> (beetles) |
| Forewings (wing covers) basal half leathery, distal half membranous, slightly overlapping at tip (bedbugs have vestigial wings), mouth parts suctorial. | <i>Hemiptera</i> (bugs) |
| Forewings moderately leathery, apical portion slightly overlapping, mouth parts distinctly mandibulate. | <i>Orthoptera</i> (cockroaches, crickets) |

the adult. In order to attain the winged condition (fleas which are wingless must also undergo this profound change) an entirely different stage is interjected into the life cycle, namely, the pupa. The young are called larvæ (maggots in flies, wrigglers in mosquitoes). In those forms in which there is no pupal stage the young are commonly called nymphs.

ANOPLURA—THE SUCKING LICE

Anoplura.—The sucking lice. The blood-sucking lice of man are *Pediculus humanus corporis*, the body louse; *P. humanus capitis*, the head louse; and *Phthirus pubis*, the crab louse.

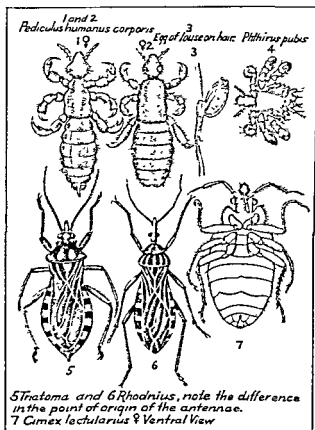


FIG. 89.—Lice, cone-nosed bugs (*Triatoma* and *Rhodnius*), and bedbug (*Cimex*).

The Genus *Pediculus*.—The body louse and head louse are indistinguishable. They are 2 to 3.5 mm. in length, grayish to sooty in color, reddish when newly engorged with blood (Fig. 89, 1 and 2). The mouth parts situated at the tip of the narrowly rounded head are distinguishable by a protrusion bearing recurved teeth. The piercing structures (stylets) are retracted into a tiny pocket when at rest. The antennæ are five-jointed. Prominent simple eyes are present. The legs are stout, the tarsi have a large curved claw, that of the anterior leg being particularly prominent and closing against a thumb-like process on the tibia. This enables the lice to hold fast to hairs. The eggs, or nits, are glued to the hairs of the head by the head louse, and those of the body louse are glued to the fibers

of clothing, especially along the seams. The eggs are oval whitish objects fitted with a cap (Fig. 89, 3). They measure about 0.9 mm in length and although visible to the naked eye, they are best detected with the aid of a hand lens.

The Genus Phthirus.—The crab louse, which is smaller than the aforementioned species, has a broad short body with prominent clawed feet, which gives it the appearance of a minute crab (Fig. 89, 4). This louse may be found on all parts of the body having coarse hair, from the eyebrows to the ankles, but it occurs most frequently in the pubic region. The eggs are glued to hairs in the region infested.

SIPHONAPTERA—THE FLEAS

The leathery bodies of fleas are greatly compressed laterally and furnished with numerous backward pointing bristles. In some species of fleas there are conspicuous rows of teeth (combs) known as *ctenidia* which are situated on the ventral margin of the head (*genal ctenidium*) and on the hind margin of the pronotum (*pronotal ctenidium*). The presence or absence of these ctenidia is of importance in identification (Fig. 90). The head is joined broadly to the small thorax. Eyes are present in some species, and absent in others; the antennæ are short and club-like lying within grooves on the sides of the head. The piercing mouth parts are relatively conspicuous and the mandibles are blade-like and serrate. The legs are long and powerful, particularly the hind pair. The coxæ are enormously developed. The abdomen possesses a characteristic "pin cushion" or saddle-like structure located dorsally near its tip, the *pygidium*. This structure, together with an internal abdominal more or less comma-shaped spermatheca (*receptaculum seminis*), visible with special treatment, in the female is used in detailed classification (see Fig. 90, lines 1, 4, 5 and 6). The size of fleas ranges from 1.5 to 4 mm. The males are as a rule smaller than the females. Although fleas show some degree of host preference they are not highly host specific. Both males and females are blood suckers.

The glistening white, minute, oval eggs are deposited either on the host animal itself from which the dry eggs drop to the ground or are deposited on the ground, floor, bedding, in rodent nests, in crevices, etc. The larvae (Fig. 90, line 2) are maggot-like, legless, sparsely haired, and very active. The head is 1 segment) is w (Fig. 90, line 1) size because of the covering of dust and lint.

Pulex Irritans (Fig. 90).—*Pulex irritans* is known as the human flea, although it will freely attack swine, dogs, rats, and other animals. It is cosmopolitan in distribution. In this species both the oral and pronotal ctenidia are absent as in *Xenopsylla cheopis* (Fig. 90), the Asiatic rat flea, an important vector of plague. The latter may be roughly distinguished from the former in that the ocular bristle is in front of and just above the middle of the eye. *Nosopsyllus* (= *Ceratophyllus*) *fasciatus* (Fig. 90) is the European rat flea, widespread over Europe and America. This species has only one set of ctenidia, the pronotal (the oral ctenidium is absent). It closely resembles the California ground squirrel flea *Diamanus montanus* (= *Ceratophyllus acutus*), but the latter may be recognized by a spine at

the tip of the second joint of the hind tarsus longer than the third joint and reaching over on the fourth joint. *Ctenocephalides canis* and *Ctenocephalides*

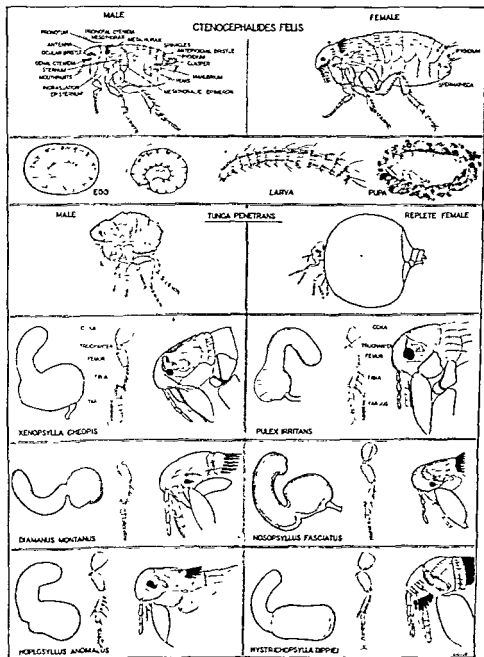


FIG. 90.—Showing stages in life cycle of a flea, also structural details used in classification of Siphonaptera. Note particularly drawings of characteristic inverted comma-shaped spermathecae at left of species indicated (Hermes' Medical Entomology, courtesy of Macmillan Company.)

felis are the dog and cat flea, respectively. They resemble each other closely. In both species both oral and pronotal ctenidia are conspicuously present.

Tunga Penetrans.—The jigger (Fig. 90), also known as chagoe or sand flea, is a minute flea only about 1 mm. in length with a conically shaped forehead, no ctenidia (combs), and has slender legs. The gravid female jigger buries itself in the skin of its host until the last abdominal segment is almost level with the surface. In this position the abdomen swells to the size of a small pea. Although man and pig are the favored hosts, they may attack cats and dogs and other animals.

TABLE 88—KEY TO COMMON SPECIES OF FLEAS*

(ORDER SIPHONAPTERA)

1. The three thoracic tergites together always shorter than first abdominal tergite 2
- The three thoracic tergites together always longer than first abdominal tergite 3
2. and femur without *Tunga penetrans*
3. *Chelidophaga gallinacea*
4. bristles 4
5. vs of bristles 9
6. 5
7. 8
8. Pronotal comb present 9
9. Mesosternite very narrow with but one internal rod-like incrasation which extends from insertion of coxa forward to anterior border *Pulex irritans*
- Mesosternite with two internal rod-like incrasations, one extending from insertion of coxa forwards and one extending upwards *Xenopsylla* 10
10. Antepygial bristle of male on a marginal cone, in female, head of spermatheca much broader than base of tail, the dark coloring of tail almost confined to swollen base *Xenopsylla brasiliensis*
- Antepygial bristle of male and female sub-marginal, head of spermatheca not, or but little, wider than tail, tail darkened to about one-half 7
7. In male ventral cone of sternite IX with dorsally and laterally transparent; in female base of tail of spermatheca at least half as broad again as head *Xenopsylla*
- In male ventral cone of sternite IX with dorsal and ventral margins equally transparent; in female base of tail of spermatheca not, or not much, broader than head *Xenopsylla cheopis*
8. First genal spine much shorter than second, head about one and a half times as long as high *Ceratophyllus*
- First genal spine about as long as second or only slightly shorter, head about twice as long as high *Ceratophyllus*
9. On inner side of mid and hind coxae longer than bristles from base to apex, eyes well developed *Pharysia*
- On inner side of mid and hind coxae longer than bristles at most in apical half 10
10. Pronotal comb with 24 or more spines, third pair of plantar bristles of tarsal segment V lateral *Ceratophyllus*
- Pronotal comb with less than 24 spines, eye not red, red *Xenopsylla*

DIPTERA—THE FLIES

Diptera. Flies (including mosquitoes, gnats, midges, etc.). The order Diptera can generally be distinguished by the fact that there is only one pair of membranous wings, the second pair is represented by a tiny pair of knobbed rod-like organs known as halteres (Fig. 87). On many of the flies related to the housefly there are present tiny eyespots at the base

* From the M. A. Society.

of each wing called *alula* or *calypteres*. The antennæ vary considerably, ranging from the conspicuous thread-like antennæ of mosquitoes and their close relatives (*Nematocera*) to the inconspicuous antennæ of houseflies and their allies (*Brachycera*). The mouth parts are profoundly modified and mosquitoes and stab e housefly and the In all Diptera the metamorphosis is complete, *i. e.*, there are the following stages in the life cycle: egg, larva, pupa, adult.

MOSQUITOES

Culicidæ.—*Adults.*—Mosquitoes are distinguished from all nematoceran Diptera: (1) by the presence of characteristic scales clothing the wing veins and wing margin (Fig. 88, *A*) and more or less abundant on the head and body; (2) the wing venation (Fig. 88, *B*). The antennæ are long and slender (plumose in the males), the mouth parts are elongate and formed for piercing and bloodsucking in the females, although not all female mosquitoes are bloodsuckers. The compound eyes are prominent. The abdomen of male mosquitoes terminates in complicated copulatory and accessory structures useful in classification (terminalia). The larval and pupal stages are always aquatic.

The *larvæ* (wrigglers) of Culicidæ (Fig. 91) are distinguished from all other dipterous larvæ by the possession of a complete head capsule and the presence of only one pair of functional spiracles (air openings) situated dorsally on the eighth (eighth and ninth fused) abdominal segment. In all but *Anopheles* mosquito larvæ the spiracles emerge within a breathing siphon which is represented only by a blunt protuberance in *Anopheles* (Fig. 91). The tenth or last segment of the abdomen terminates in anal gills and brushes. The larvæ of *Anopheles* lie suspended parallel to the water surface by means of palmate hairs (Fig. 91) while *Culex* and *Aedes* species hang with head downward and the body at an angle with the water surface (Fig. 91).

The *pupæ* (tumblers) (Fig. 91) are comma-shaped and non-feeding but very active. The arched anterior portion comprises the head and thorax, the tail portion (abdomen) terminates in a pair of swimmerets. The breathing tubes (one pair, also called trumpets) are situated in the mid-thoracic region. In *Anopheles* the breathing trumpets are relatively short, *es* species the trumpets are

ylindrical or spindle-shaped; some are smooth, others are reticulated or furnished with floats. *Culex* and closely related genera lay their eggs in rafts (Fig. 91); *Anopheles* lay their ornamented eggs singly, often arranged in geometrical figures (Fig. 91); *Aedes* and *Psorophora* lay their eggs singly (Fig. 91), frequently on the soil where there may be no water and remain (often over winter) until water is supplied by nature—rain, melting snow or tidal action as in the salt marsh mosquitoes.

Genus *Anopheles*.—About 200 anopheline species are known for the whole world, of which 63 occur in the New World. Excluding *Bironella*, which is allied to *Anopheles*, the female palpi are nearly as long as the proboscis (Fig. 91). In the males the palpi are longer than the proboscis

and dark areas. The posterior narrow segment of the thorax, the *scutellum* (Fig. 91), is raised and crescent-shape (rounded) in all Anophelini except *Chagasia*, in which the scutellum is trilobed as in *Culex* and *Aedes*.

IMPORTANT VECTORS OF MALARIA¹

United States:

A. maculipennis freeborni Aitken; *A. quadrimaculatus* Say.

Mexico and Central America:

A. albimanus Wiedemann; *A. darlingi* Root; *A. pseudopunctipennis pseudopunctipennis* Theobald; *A. punctimacula* Dyar and Knab.

Caribbean area:

A. albimanus Wiedemann; *A. aquasalis* Curry (= *tarsimaculatus* in part); *A. bellator* Dyar and Knab.

South America:

A. albimanus Wiedemann; *A. albitarsis* Lynch Arribalzaga; *A. darlingi darlingi* Root; *A. gambiæ* Giles (exterminated?); *A. pseudopunctipennis pseudopunctipennis* Theobald.

Persian Gulf and Caucasian area:

A. sacharovi Favr (= *elutus*); *A. stephensi* Liston; *A. superpictus* Grassi.

Afghanistan, Baluchistan, India, Ceylon:

A. annularis van der Wulp (= *fuliginosus*); *A. culicifacies* Giles; *A. fluriatilis* James; *A. jeyporiensis* James; *A. minimus* Theobald; *A. philippinensis* Ludlow; *A. stephensi* Liston; *A. sundaicus* Rodenwaldt; *A. superpictus* Grassi; *A. taruna* Iyengar.

Burma, Malaya, Indo-China, Thailand, South China, Formosa:

A. aconitus Donitz; *A. culicifacies* Giles; *A. hyrcanus sinensis* Wiedemann; *A. maculatus* Theobald; *A. jeyporiensis candidiensi* Koidzumi; *A. minimus* Theobald; *A. sundaicus* Rodenwaldt; *A. umbrus* Theobald.

Europe:

A. labranchiæ atroparvus van Theil; *A. labranchiæ labranchiæ* Falleroni; *A. messeæ* Falleroni; *A. sacharovi* Favr (= *elutus*); *A. superpictus* Grassi.

North Africa, Middle East:

A. claviger Meigen (= *bifurcatus*), *A. labranchiæ labranchiæ* Falleroni; *A. multicolor* Camboulin; *A. pharænsis* Theobald; *A. sacharovi* Favr (= *elutus*); *A. sergenti* Theobald; *A. superpictus* Grassi.

Central and South Africa:

A. funestus Giles; *A. gambiæ* Giles; *A. hancocki* Edwards; *A. hargreavesi* Evans; *A. moucheti* Evans; *A. moucheti nigeriensis* Evans; *A. nui* Theobald; *A. pharænsis* Theobald; *A. pretoriensis* Theobald.

¹ For the identification of anopheline mosquitoes consult "The Anopheline Mosquitoes of the World—a Guide to Their Identification, Distribution, Habits, and Relation to Malaria," prepared for The Preventive Medicine Division, Office of The Surgeon General, U. S. Army, by Paul F. Russell, Lieutenant Colonel, Medical Corp., U. S. A., Lloyd E. Rozeboom, B.S., Sc D., and Alan Stone, B.S., Ph D. Published by The American Entomological Society at the Academy of Natural Sciences, Philadelphia, 1943.

Other Genera.—*Psorophora ciliata* is a large and vicious mosquito known as the American gallinipper. *Theobaldia inciens* is a common western American spotted-winged culicine mosquito. *Mansonia annulifera* is an important vector of *Wuchereria malayi* in India and Ceylon. This genus is particularly interesting, in that the eggs are deposited on the under-surface of leaves of aquatic plants and the larvæ have a breathing tube terminating in a spine which enables them to pierce underwater vegetation and draw air from them, and hence do not come to the surface to breathe. *Armigeres obturbans* has been incriminated as a vector of dengue in Formosa. *Hæmogogus capricornii* has been found naturally infected with yellow fever virus (jungle yellow fever) in the forests of Brazil where it breeds in collections of water in treeholes high up in the treetops where it has been aptly said to "associate with the monkeys in the interlacing branches high above the jungle floor."

SANDFLIES, BITING MIDGES, BUFFALO GNATS, EYE GNATS

The Sandflies.—Sandflies belong to the family Psychodidae, genus *Phlebotomus*. To this family also belong the non-biting mothflies (*Psychoda*) which often swarm in and about sewage disposal plants, cesspools, and even wash basins. The *Psychoda* larvæ are commonly found in the filter beds of sewage disposal plants. Unlike these latter flies, though system-

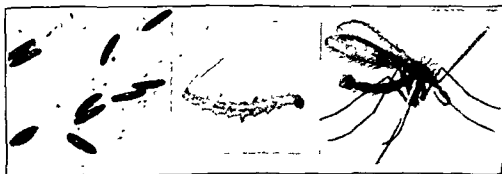


FIG 92.—*Phlebotomus verrucarum* (sandfly). Eggs, larva, adult male. (From Marshall Hertig, Am Jour. Trop. Med., courtesy of Williams & Wilkins Company.)

atically related, are the *Phlebotomus* flies or sandflies (Fig. 92) which are bloodsucking. Like other members of the family, their bodies and wings are quite hairy. The antennæ are long and filamentous, consisting of 12 to 16 segments. The wings when at rest are held erect over the body. These gnats are very tiny, usually from 3 to 5 mm. in length. *Phlebotomus papatasi* is the vector of a dengue-like disease known as pappataci fever. *Phlebotomus verrucarum* is the vector of verruga peruviana. *P. chinensis*, *P. perniciosus* and other species are vectors of visceral leishmaniasis (kala-azar), while *P. papatasi* and *P. sergenti* and other species are vectors of cutaneous leishmaniasis (Oriental sore).

Biting Midges.—Members of the family Ceratopogonidae, belonging to the genera *Culicoides*, *Ceratopogon* and *Leptoconops*, are commonly called "punkies" or "no-see-ies" (Fig. 93). They may conveniently be divided into two groups: the males do not bite) in contrast to the females, which do bite, and the females belonging

Simulium damnosum and *S. neari* are vectors of *Onchocerca volvulus* in tropical Africa, and *S. metallicum*, *S. ochraceum* and *S. callidum* are vectors of the same worm in Guatemala and southern Mexico. *S. meridionale* is known as the turkey gnat and is a serious pest of turkeys, domestic animals and man throughout the southern United States.

Hippelates Flies.—Eye gnats (*Hippelates* flies) belong to the Chloropidæ, a family of tiny (2 mm.) non-bloodsucking flies which are attracted to perspiration, excretions, exudations of sores, and particularly lachrymal secretions of man and domestic animals. They are therefore dangerous mechanical vectors of eye infections and infections of the skin, such as yaws. In the genus *Hippelates* the hind tibiae bear a distinct curved, shiny black apical or subapical spur.

BLOODSUCKING FLIES

Family Tabanidæ.—This family of Diptera includes a large number of species of heavy bodied but swiftly flying bloodsuckers, commonly known as horseflies, gadflies, greenheads, breeze flies or deer flies. They are notorious as pests of horses, cattle and deer. They are strictly day biters, i. e., diurnal in feeding habits. Some of the larger species are a good inch and a half in length while some of the smaller species are barely half an inch long. The compound eyes are large, the antennæ are short and generally porrect, consisting of three joints; the terminal segment is somewhat thorn-like. There is no arista. The mouth parts are short and blade-like. The males are not bloodsuckers, but feed on plant juices.

All tabanids breed in water or marshy situations where the eggs are deposited on leaves of aquatic vegetation or leaves of overhanging trees. The spindle-shaped larvæ feed on earthworms and soft bodied aquatic animal life. To pupate, the larvæ crawl out of the mud to drier situations. The entire life cycle commonly requires a year, although a few tropical species may have two or more generations a year.

Genus Tabanus.—Members of this genus are robust, with clear or smoky wings. *Tabanus atratus* is the black horsefly and measures better than 1½ inches in length. It occurs in many parts of the United States east of the Rocky Mountains. *Tabanus stygius* is the "black and white horsefly" which measures about 1 inch in length and occurs in the United States east of the Rocky Mountains, while *Tabanus punctifer* is the "black and white horsefly" of the Pacific Coast. *Tabanus ricarius* (*T. costalis*) is the common "greenhead," a serious stock pest of the south. *Tabanus striatus*

other as
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Genus Chrysops.—These are more robust than the *Tabanus* and are conspicuously banded with smoky areas. The antennæ are similar to those of the previous genus but more conspicuous; the hind tibiae bear spurs at the apical end. *Chrysops dimidiata* and *C. silacea* (mango flies) are vectors (intermediate hosts) of the human filarial worm, *Loa loa*, in tropical Africa. *Chrysops discalis* transmits tularemia mechanically in Utah and adjacent states.



Glossina palpalis inhabits the tropical rain forest belt of Africa, chiefly the Congo and West Africa, where it feeds on the blood of mammals, crocodiles and large lizards. It is the chief vector of the causal organism of Gambian trypanosomiasis (sleeping sickness), *Trypanosoma gambiense*.

Glossina morsitans inhabits the high savannah area of Rhodesia, the Sudan and the Belgian Congo where it feeds on large game animals. It is typically a "game fly." It is the principal vector of *Trypanosoma rhodesiense*, causal organism of Rhodesian trypanosomiasis (Rhodesian sleeping sickness).

Glossina swynnertoni is a strong vector of Rhodesian sleeping sickness as well as nagana of cattle and sheep. It is a fly of the driest and most open areas where it is essentially a "game fly."

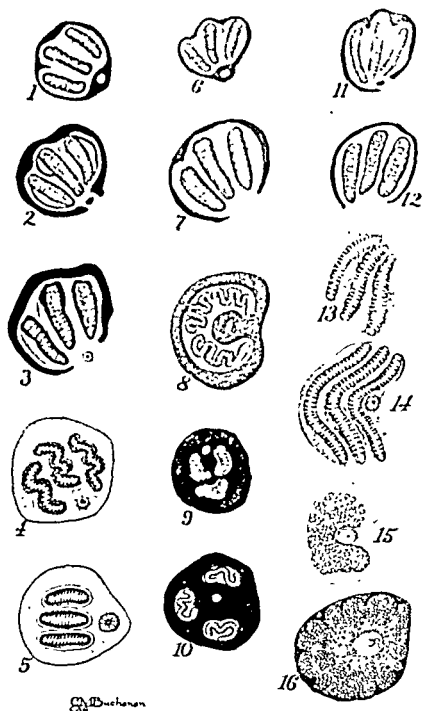
NON-BITING, MYIASIS-PRODUCING FLIES

Family Muscidae.—The most important member of the family Muscidae is the common housefly, *Musca domestica*, which is cosmopolitan in its distribution. This fly occurs in various shades of black, gray and yellowish, usually with four dark longitudinal stripes on the pronotum. It measures from 4 to 7.5 mm. in length. The compound eyes are prominent. This fly is prone to lay its eggs in horse manure, but will readily oviposit in almost any kind of dung and decomposing vegetable and animal matter. The larvæ are typical maggots and when fully grown may measure from 7 to 10 mm. in length, are pointed anteriorly and truncate and rounded posteriorly, pale yellow to whitish in color. The posterior spiracular areas are D-shaped (Fig. 96). Larvæ may be ingested with food and may cause intestinal myiasis.

Family Anthomyiidae.—Although this family includes many species of flies whose maggots infest growing vegetables, such as onions, beets and spinach, cabbage and other crucifers, it also includes the "lesser housefly," *Fannia canicularis*, and the "latrine fly," *Fannia scalaris*, whose spined larvæ breed in dung and decayed animal and vegetable matter. Their larvæ when swallowed may produce accidental intestinal myiasis.

Family Calliphoridae.—To this family belong the blow flies, bluebottle and greenbottle flies and related species. They range in size from 5 to 17 mm., are sombre black, dull gray or brilliant metallic blue or green with gold, brass and coppery iridescence. They are typically muscoid as are the larvæ, which have nearly circular spiracular plates with three nearly longitudinal slits converging slightly inward (Fig. 96). The maggots develop in garbage, fresh or decaying animal matter or excreta and may be ingested with food, causing accidental intestinal myiasis. Some species may cause traumatic myiasis, or they may invade open body cavities, particularly the nose.

Calliphora erythrocephala and *C. vomitoria* are common bluebottle flies of Europe and America; *Lucilia cuprina* and *L. sericata* are greenbottle flies; *Cochliomyia americana* is the screw worm fly; *Cochliomyia macellaria* closely resembles *C. americana* but is scavenger in habit; *Phormia regina* is the "black blowfly"; *Pollenia rudis* is known as the "cluster fly"; the larvæ of *Protocalliphora azurea* occur in birds' nests and suck blood from the nestlings; *Auchmeromyia luteola* is the "Congo floor maggot" and *Cordylobia anthropophaga* is the "tumbu fly," the larvæ of both species attacking man in Africa.



C. Buchanan

FIG. 96.—The posterior spiracular pattern of representative fly larva (right spiracular plate only). 1, *Calliphora vomitoria*; 2, *Chrysomya bezziana*; 3, *Cochliomyia macellaria*; 4, *Cordylomyia anthropophaga*; 5, *Auchmeromyia luteola*; 6, *Lucilia sericata*; 7, *Phormia regina*; 8, *Musca domestica*; 9, *Muscina stabulans*; 10, *Stomoxys calcitrans*; 11, *Sarcophaga fuscicauda*; 12, *Wohlfahrtia vigil*; 13, *Dermatobia hominis*; 14, *Gasterophilus intestinalis*; 15, *Hypoderma bovis*; 16, *Cestrus oris*. (Partly original and partly adapted from various sources*) (Craig and Faust, Clinical Parasitology, courtesy of Lea & Febiger.)

Family Sarcophagidæ.—The members of this family are typical flesh flies or scavenger flies. They are medium sized to large flies, bristly and hairy, usually with dark longitudinal pronotal stripes; the eyes are red and large; the wings and squamæ are large. They are larviparous. The larvæ are typically muscoid; the posterior spiracles nearly circular in a deep depression, each with three nearly parallel slit-like openings (Fig. 96). *Sarcophaga carnaria* is the gray blowfly of Europe, and *Sarcophaga hæmorrhoidalis* is common in North America; *Wohlfahrtia magnifica* larviposits in wounds, causing traumatic and cutaneous myiasis and myiasis of the ears, nose and eyes.

Family Œstridæ.—The bot and warble flies. This family includes the sheep botfly *Œstrus ovis*, which larviposits in the nostrils of sheep and goats. The spiny larvæ of *O. ovis* are commonly found in the eyes of sheep herders.

Family Cuterebridæ.—This is a family of robust bot flies, of which *Dermatobia hominis* is an important member. The adult fly is about 15 mm. in length, with vestigial mouth parts, dull bluish-gray thorax, bluish-green abdomen and dull-brown wings. It is found in the tropics and subtropics of America. The female attaches her eggs to the abdomen of mosquitoes and other dipterous insects and even ticks, where they remain until the end of incubation and until an opportunity offers for the larva to transfer itself to the skin of a host such as pig, dog, monkey, man or other animal. The larval development then takes place entirely in the subcutaneous tissues, where a tumorous swelling is produced with a small opening through which the larva obtains air. The larvæ are plump and ovoid, similar to ox warbles. When full grown the larvæ leave the tumorous swellings, drop to the earth where they pupate; the pupal stage lasts from three to six weeks. Members of the genus *Cuterebra* infest rabbits and rodents.

Family Hypodermatidæ.—This family includes the warble flies (heel flies) of cattle and deer. A form of creeping myiasis in man is caused by larvæ of these flies. *Hypoderma lineata* is a cosmopolitan species, while *Hypoderma boris* is less widely distributed.

Family Gasterophilidæ.—This family includes the horse bot flies. The larvæ infest the stomach and intestine of horses. The eggs (or larvæ in certain species) are deposited on the hairs of the belly, legs and around the mouth and the larvæ are introduced into the mouth by licking. The

G. nasalis, and *G. hæmorrhoidalis*—all horse bots.

HEMIPTERA (BUGS) OF MEDICAL INTEREST

Order Hemiptera (Heteroptera).—In the Hemiptera the head is usually prognathous, generally free and often with a neck-like region. The compound eyes are large and prominent, a pair of ocelli is usually present; the antennæ are usually well developed and 4 to 5-jointed; the mouth parts are modified for piercing; the wings (two pairs) are usually folded flat over the dorsum, with the apical portions overlapping the forewings

(wing covers); the basal portions thickened or leathery and the apical portion membranous. The hind wings are wholly membranous. In the bedbugs, a member of this order, the wings are absent except for the merest

rostrum three-segmented and resting in a ventral groove, true wings are absent and the wing covers are represented only by mere pads; the legs are short. *Cimex lectularius* (Fig. 89, 7) is the common cosmopolitan bedbug which attacks man. A second species, *C. hemipterus*, occurs in the tropics. Neither is of any practical importance as vectors of disease organisms.

Family Reduviidae.—Members of this family are commonly known as conenoses, kissing bugs and assassin bugs. The head is narrow, longer than broad, pointed anteriorly with neck free; antennae filiform, apical segments often very fine and frequently broken off, four or five-segmented, eyes well developed, near middle or at base of head; rostrum curved, three-segmented with tip resting in a furrow between the coxae. The wings are typically hemipteran, and most of the species are good fliers.

Genus Triatoma.—Members of this genus (Fig. 89, 5) are found in nearly all the warmer parts of the world. Many of the species are partially predaceous, while others are practically entirely bloodsucking. Many feed on a wide variety of hosts but some species live only in the nests of wild rodents. The head is elongated in front of the eyes; the antennae are inserted about midway between the eyes and apex of head; the thorax is constricted anterior to middle, and the posterior angles are rounded, they are black or brown, with red or yellow markings.

Triatoma megista.—The "barbeiro" of Brazil, Peru and other South American countries, measures from 2.5 to 3.3 cm. in length, is a black species marked with red. It lives mainly in the huts of natives, where it feeds on the inhabitants at night. Both sexes are bloodsucking. The females lay their eggs in small batches in crevices in the walls and dusty corners of houses. The newly hatched nymphs resemble the adults except for the absence of wings. There are five molts before the adult stage is reached, the whole life cycle requiring about one year. This species is a potent vector of *Trypanosoma cruzi*, cause of Chagas' disease.

Other species of *Triatoma* are *T. dimidiata*, a vector of Chagas' disease in Panama; *T. sanguisuga*, a widely distributed North American species, commonly gets its blood meal second-hand by feeding on bedbugs—it is sometimes called the "Mexican bedbug;" *T. protracta* and *T. uhleri* are black species of California and the southwestern United States and inhabit the nests of wood rats (*Neotoma*).

Genus Rhodnius.—In this genus (Fig. 89, 6) the antennae are inserted near the apex of the head and the second joint of the beak is more than twice as long as the others. *Rhodnius prolixus* has habits similar to those of *Triatoma megista* and takes its place as vector of *Trypanosoma cruzi* in Venezuela and Colombia. It is reported that this bug ingests the liquid feces of other bugs, which makes it even a more potent factor in the spread of the infection.

TICKS, MITES, SPIDERS AND SCORPIONS

Class Arachnida.—To this class belong the ticks, mites, spiders, scorpions, solpugids and whip scorpions. In the arachnids the body shows a strong fusion: the *cephalothorax* (comprising the head and thorax), and the *abdomen* (Fig. 97). In the ticks and mites there is a fusion of both the cephalothorax and abdomen to form a sac-like body. Arachnids are never winged, and the adults have four pairs of legs except that the larvæ of ticks and mites have but three pairs, the fourth pair being acquired with the first molt. Eyes when present are simple; the mouth parts consist of a pair of pincer-like *chelicerae*, and a pair of simple segmented pedipalps. In the Acarina there is a *hypostome*. The legs typically have six or seven segments which, beginning next to the body, are named as follows: (1) coxa; (2) trochanter; (3) femur; (4) tibia; (5) protarsus; and (6) tarsus. All forms of arachnids deposit eggs except the scorpions and some mites which are viviparous. Development from the young to adult is gradual, involving a series of molts. The respiratory system of ticks and mites is tracheal as in insects except that there is usually but one pair of spiracles; in spiders it consists of a combination of lung book and tracheæ. There is frequently a strong sexual dimorphism; the males are commonly smaller than the females.

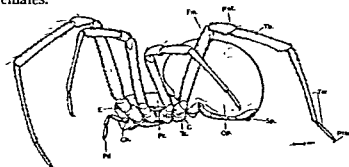


FIG. 97.—Showing external morphology of a spider. *C*, coxa; *Ch*., chelicera; *E*., eyes; *Fm*, femur; *Op*, opisthosoma; *Pat*., patella; *Pd*., pedipalp; *Pr*., prosoma; *Ptar*., pretarsus; *Sp*., spinnerets; *Tar*., tarsus; *Tb*., tibia; *Tr*., trochanter. (Hermes' Medical Entomology, courtesy of Macmillan Company.)

TABLE 89.—KEY TO TERRESTRIAL ORDERS OF ARACHNIDA*

1.	2
2.	3
3.	4
4. Palpi chelate (pincer-like)	Scorpionida (scorpions)
Palpi not chelate	Pedipalpida (whip scorpions)
5. Abdomen constricted	Pseudoscorpionida (false scorpions)
Abdomen not constricted	5
6. Legs very long and slender, body hairless, whole body fused together	
Legs moderate; body usually hairy, appearing 3-segmented	Phalangida (harvest spiders)
7. Abdomen constricted at base and joined to cephalothorax by a narrow stalk	Solpugida (solpugids)
Abdomen fused with cephalothorax	Araneida (true spiders)
	Acarina (ticks and mites)

* Adapted after various authors.

Arachnid Metamorphosis.—In the Arachnida (ticks, mites, spiders, scorpions) the adult form is attained after a simple metamorphosis, *i. e.*, there is a series of molts and with each an increase in size follows.

TICKS AND MITES

Order Acarina.—This order comprises the ticks and the mites which range in size from microscopic mites to certain ticks which may be $\frac{1}{4}$ inch in length. They show a strong fusion of the body parts, becoming strikingly sac-like and leathery, particularly in the ticks.

The Ticks

Super-family Ixodoidea.—In the ticks the mouth parts, together with the *basis capituli*, form a structure known as the *capitulum*. The mouth parts consist of a pair of protrusible *chelicerae* and a structure known as the *hypostome* which bears recurved denticles. A pair of simple eyes may be present; however, many species are eyeless. A pair of spiracles (breathing openings) are situated lateroventrally on the abdomen, one on each side near the third and fourth coxae. The females are capable of great distension, and when fully engorged are seed-like in form.

The ticks are commonly divided into two groups, the hard bodied ticks (*Ixodidae*) and the soft bodied ticks (*Argasidae*). The hard bodied ticks (Fig. 98) have a hard shield (*scutum*) on the dorsum of the body immediately posterior to the capitulum; in the male this dorsal shield (*scutum*) covers the dorsum of the body; the mouth parts are terminal. When biting these ticks usually remain attached to the host for several hours to several days. In the soft bodied ticks (Fig. 98) the scutum is absent and the mouth parts are anteroventral. When biting they usually remain attached for an hour or less. Both male and female ticks of both groups suck blood. The hard bodied ticks are either one, two, or three hosted, commonly the latter, in which three host animals, either the same individual or even different species, are required for complete development. Each feeding stage—seed tick (larva), nymph and adult, requires a separate host and after full engorgement drops to the ground and in the case of the seed tick and nymph there is a molt. When the adults drop to the ground (frequently in copulation) the females lay their eggs and shortly thereafter die, as do the males. Ticks of the genus *Dermacentor* are good examples of three-hosted ticks. *Boophilus annulatus*, the vector of Texas cattle fever, is a one-host tick, hence completes the development of the feeding stages on the same bovine host.

The soft bodied ticks are many hosted, *i. e.*, five or more hosts may be needed to complete the cycle. There is usually a molt after each feeding, and molts may occur after maturity is reached between egg layings and further feeding.

Hard Bodied Ticks.—*Dermacentor andersoni* (Fig. 98) is the wood tick of the Rocky Mountain region of the United States and also parts of California, Oregon, Washington, and British Columbia. The adult ticks feed mostly on large animals such as horses, cattle, sheep, deer and coyote; the larvae and nymphs prefer rodents such as ground squirrels, woodchucks and chipmunks. All three stages may feed on animals of intermediate size such as jack rabbits, badgers and marmots. This species is a typical three-

host tick. The normal life cycle is two years. It is the vector of *Rickettsia rickettsi*, the causal agent of Rocky Mountain spotted fever in western North America. It is also a vector of tularemia, Colorado tick fever, and may cause tick paralysis.

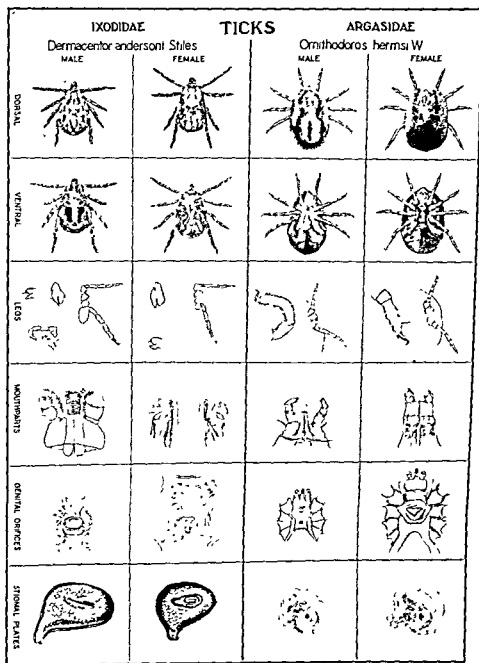


FIG. 98.—Showing structural details used in classification of ticks. Left, Ixodidae (hard bodied ticks), right, Argasidae (soft bodied ticks). (Hermes' Medical Entomology, courtesy of Macmillan Company)

Dermacentor variabilis, the common dog tick, is a vector of Rocky Mountain spotted fever in the eastern United States. It is a three-host

tick and may complete its cycle in one season. The larval stage is largely restricted to field mice.

Hæmaphysalis leporis-palustris is a vector of the rickettsiæ of Rocky Mountain spotted fever from rabbit to rabbit in nature, thus maintaining the endemicity of the infection.

transmits the causal organisms

It is a one-hosted tick.

Paulo fever (*Rickettsia brasiliense*)
to man in South America.

Amblyomma americanum, the "lone star" tick, is a vector of Rocky Mountain spotted fever in Texas and Oklahoma.

Soft Bodied Ticks.—*Ornithodoros moubata* of Central Africa is the only member of this genus residing strictly in human habitations and transmits the spirochete of relapsing fever from man to man. It also feeds on domestic animals.

Ornithodoros rudis of Panama and South America is an important vector of relapsing fever, as is *O. turicata* in Texas, New Mexico, Oklahoma, Mexico and elsewhere.

Ornithodoros hermsi (Fig. 98) inhabits the nests of chipmunks in various parts of California (also Nevada, Idaho, Oregon) at elevations of 4000 feet and above. It is naturally infected with the spirochete of relapsing fever, picking it up when sucking blood from its natural host and transferring it to man. The spirochete is transmitted congenitally from the adult female tick to her offspring. This tick is a small species hardly larger than a bedbug.

Ornithodoros parkeri transmits the infection in Utah, Montana, Idaho, Or. on burrowing rodents.

ick." It invades the ears of cattle, horses, mules and various other animals, also man. It is widely distributed in subtropical parts of the United States, Mexico, Central and South America, South Africa and India.

The Mites

With the exception of certain free living species, mites are minute, many of them barely visible to the naked eye. Like the ticks, the parts of the body are fused, giving a sac-like or even worm-like appearance to the body. The mouth parts, which are quite varied, follow the general pattern of ticks. They bear four pairs of legs except in the larval stage, when there are but three pairs. Nearly all species deposit eggs; however, there are some ovoviviparous forms.

Genus Sarcoptes.—Minute burrowing mites, oval in form, with stubby legs terminating in pediculate suckers at the tip of the first two pairs. The female mite burrows into the skin and lays her eggs, numbering from 10 to 25 as in tortuous galleries, eventually dying in the blind end of the channel. The eggs hatch in three or four days and the larvae repeat the burrowing in a tangential direction, reaching maturity in another seven to ten days. Intense itching usually begins about ten days after infection. These mites are commonly called itch mites. The species infesting man is known as *Sarcoptes scabiei* var. *hominis* (Fig. 99),

and produces the condition known as scabies. Transmission of these mites from person to person normally takes place by actual contact. The point of invasion is where the skin is thin, as between the fingers,

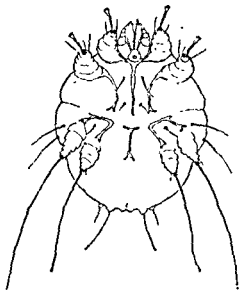


FIG. 99.—Mite causing scabies or itch, *Sarcoptes scabiei*. (Herna's Medical Entomology, courtesy of Macmillan Company.)

on the penis, or in the bend of the knee or elbow. The diagnosis of scabies may be proved by demonstrating the mites or their eggs in skin scrapings taken at the site of a lesion and cleared in 10 per cent potassium hydroxide.

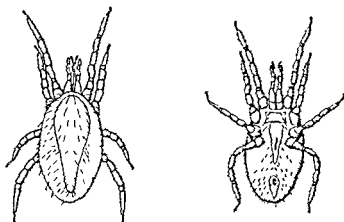


FIG. 100.—The tropical rat mite, *Liponyssus bacoti*. Dorsal view, left; ventral view, right. (After Stewart)

Genus *Liponyssus*.—The tropical rat mite, *Liponyssus bacoti* (Fig. 100), occurs throughout the southern and Pacific coast region of the United States. It is frequently troublesome to man in rat-infested buildings, especially when deprived of its rat host. The mites suck blood and drop off the host after each meal. The bites produce an unusually severe dermatitis.

Experimental evidence indicates that this mite can be a vector of endemic (murine) typhus from rat to rat.

Trombicula akamushi transmits (in the larval stage) a typhus-like disease of Japan known as Japanese river fever, also called Japanese flood fever, or Tsutsugamushi fever caused by *Rickettsia orientalis*. In Sumatra and Malaya a *deliensis* is believed

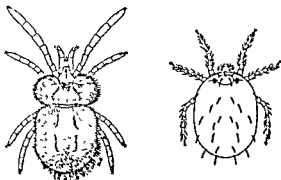


FIG. 101.—The chigger (mite), *Eutrombicula alfreddugesi*. Adult mite, left; larva, right (From Ewing's Manual of External Parasites, courtesy of Charles C Thomas, Publishers)

SPIDERS, SCORPIONS AND CENTIPEDES

Spiders.—Spiders (Fig. 97) belong to the Order Araneida, in which the cephalothorax (prosoma) is uniform and joined to the abdomen (opisthosoma) by a pedicel. The prosoma bears four pairs of legs. The chelicerae are prominent and are connected with poison glands. The pedipalps are six-segmented and leg-like. The abdomen bears not more than four, usually three, pairs of spinnerets. The very large hairy spiders of the arid southwest commonly, though erroneously, known as tarantulas (Family Aviculariidae), are fearsome in appearance but are not considered dangerous, although the bite of the much smaller black widow spider is dangerous.

Latrodectus mactans (Family Therididae) is the black widow, shoe-button, or

distinct brick markings. It is widely distributed in both North and South America, particularly the subtropical and tropical portions. It is not uncommonly found in wood piles, vacated rodent burrows, outbuildings, barns, privies, under low bridges, in culverts, etc.

Other dangerous spiders are *Latrodectus curacatiensis* (South America and West Indies), *L. geometricus* (West Indies), *L. hasselti* (Australia),

L. guttatus (Europe), *L. lugubris* (Russia), *L. menardi* and *Glyptocranium gastera* of South America.

SCORPIONS

Scorpions are easily recognized by the presence of a long fleshy five-segmented tail-like postabdomen terminating in a bulbous sac and prominent sting. The pedipalps are greatly enlarged to form powerful lobster-like jaws. There are four pairs of terminally clawed legs throughout life. The females are viviparous. Scorpions are found commonly in tropical and subtropical countries. They are nocturnal, remaining hidden during the day beneath loose stones, loose bark of fallen trees, boards, piles of lumber under floors of outbuildings and débris. *Centruroides suffusus* is the so-called Durango scorpion of Mexico. *Buthus quinquestriatus* is a dangerous Egyptian scorpion.

CENTIPEDES

Centipedes are provided with poison glands at the base of the first of the two pairs of mandibular legs where are situated fang-like claws. The large forms of centipedes may reach 10 inches in length. They bear one pair of appendages on each segment, the terminal pair being sharp and dragging. Their bites may be painful but are not dangerous. Among the more formidable looking species are *Scolopendra heros* of the southern United States, and *S. viridis* of Mexico.

PRESERVATION AND SHIPMENT OF SPECIMENS

Specimens for museum purposes or storage must be protected from fungus attack and attack by dermestid beetle larvæ and other insects. Various chemicals, such as naphthalene flakes and paradichlorobenzene, are useful to repel attack if put into small bags and pinned solidly in a corner of insect boxes or scattered among papers containing insects. Storage should be in damp-proof containers or compartments.

In shipping specimens of insects for identification they must be so packed that the appendages are not damaged. In shipping mosquitoes do not use cotton to protect the individual insects; use cellu-cotton, cotton wadding, cotton linters or lens paper. Pack lightly in a pill box. A disc of lens paper, size of the box, may be laid in the bottom. A few mosquitoes may be placed lightly on this, with another disc laid on top and thus possibly two or three layers; then fill in space with crumpled lens paper. Large flies may be pinned with insect pins (through the thorax) and the pin firmly stuck into a slab or cork fitted to a box, the size depending upon the number of insects to be shipped. For mailing, the boxes should be carefully packed—suitable mailing cases are convenient.

Small insects, such as fleas, gnats, midges, ticks, mites, mosquito larvæ and the like, should be placed in a vial of 70 per cent alcohol with 5 per cent glycerol and 1 per cent formaldehyde. The vial for shipment should be almost filled, about five-sixths full of solution and placed, well surrounded in cotton, in a mailing tube. This amount of solution prevents breaking of the specimens. If alcohol is not available 4 per cent formaldehyde may be used, with or without glycerol.

All specimens should bear a label with such necessary data as locality, host, date, collector's name, local environment and any other pertinent information.

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PART IX

Pathology

CHAPTER XXXIX

THE AUTOPSY

By JAMES E. ASH

THE primary purpose of an autopsy is to determine the cause of death more definitely than is possible by clinical methods, but its function does not cease there. Every dead body, no matter how obvious the cause of death may be, contains something of interest or value to the study of pathology. Considerable responsibility, therefore, rests on those in a position to obtain and perform autopsies to see that these opportunities are not lost. The record of the case is not complete unless it includes every noteworthy feature, both clinical and pathologic, no matter how irrelevant to the cause of death it may seem at the time. If the prosecutor will bear in mind that his is the final opportunity to gather the data from the case, and that his record is all that those coming after him will have for any study they may want to make, he is less liable to lose sight of his great responsibility.

The autopsy has additional significance in the Army from the administrative standpoint. It is not sufficient merely to determine the cause of death but it is also necessary to elicit every bit of evidence that would fix the responsibility for the death. It is only thus that the interests of the government can be protected on the one hand and that justice be done to the patient on the other. It is not sufficient, therefore, simply to determine the cause of death. Every autopsy should be a complete one, even where the cause of death is as obvious as in an airplane crash. Many important data have been lost because of superficially conducted autopsies.

I. Records.—The protocol should be as complete as possible, so that one reading it subsequently can reconstruct the picture as it was, and make his own interpretation. Descriptions, therefore, should be objective rather than by diagnoses. These latter and interpretations should be stated under comments. Sufficient copies of the protocol should be prepared to meet the requirements of the local hospital and those of the histopathologic centers and of the Army Medical Museum.

Table 60 is a satisfactory outline for a protocol. The first page should give all statistical data, the clinical diagnoses, the cause of death as determined after autopsy and the pathologic diagnoses. This is of great assistance in reviewing a number of cases. An abstract of the clinical history must be attached.

TABLE 90.—OUTLINE FOR AUTOPSY PROTOCOL

Name:	HOSPITAL	Adm. No.:	Autopsy No.:
		Rank:	A.S. No.:
Age:	Sex:	Race:	Length of Service:

Date of Admission:

Date and Hour of Death:

Date and Hour of Autopsy:

Clinical Diagnoses:

Gross and Microscopic Diagnoses:

1. _____

2. _____ (etc.)

(The above should be on the first page of the protocol)

External Examination:

Primary Incision:

Lungs:

Heart: Aorta: Splanchnic Vessels:

Spleen:

Liver:

Pancreas:

Adrenals:

Gastro-intestinal Tract:

Kidneys.

Bladder

Prostate and Vesicles:	} Or Uterus and Adnexa
Testes and Epididymes:	

Skull:

Pituitary. Brain: Cord:

Eye: Ear:

Thyroid. Parathyroids:

Bone Marrow: Muscles.

Smears: Cultures.

Chemical Examinations:

Microscopic Examination:

Comments particularly on Pathogenesis:

Witnesses:

(Signature)

Name (typed) and Rank
Prosecutor

II. Materials.—The following materials are required to perform a satisfactory autopsy:

1 heavy prosecting knife	1 pr. tissue forceps, toothed
1 amputating knife	2 probes; 1 fine, 1 coarse
1 scalpel	4 prs. heavy hemostats
1 pr. rib shears	1 steel rule
1 enterotome	2 needles, double curved, No. 1
1 pr. 6-inch scissors	Waxed string
1 pr. fine-pointed scissors	Scales and weights (metric preferred, weighing at least up to 2000 gm.)
(1 probe point preferred)	2 large sponges
1 saw (postmortem or hack saw)	Cutting board
1 chisel, 4 or 5 inches long	Waterproof apron
1 mallet, wooden	Rubber gloves
1 steel hammer with hooked handle	2 large basins or buckets
1 rachiotome or 1 pr. Brunetti's chisels for opening spine	Bunsen burner or alcohol lamp
1 pr. dissecting forceps, 5 inches	Capillary pipets, sterile
4 chemically clean jars for specimens for chemical analysis	1 syringe and needle, sterile
	1 heavy spatula for searing tissues

This list may be amplified indefinitely but with the items given one will be able to meet almost any contingency that may arise.

III. Technic.—Either the evisceration or the organ by organ method may be used, depending on the training and experience of the prosector. The details of both methods are available in standard texts on autopsies and need not be repeated here. The so-called "Y" incision is preferable when the neck and mouth structures are to be examined but it adds to the difficulty of sealing the body and for ordinary purposes the straight incision from suprasternal notch to symphysis is preferable.

IV. Postmortem Bacteriology.—Cultures should be made from heart's blood, spleen, liver, and such other organs or lesions as indicate bacterial infection. Specimens are obtained from the parenchyma by searing the surface with a heated spatula and plunging through this area a sterile capillary pipet or a sterile syringe needle.

V. Restoration of Body.—Great care must be exercised to prevent disfigurement of the body and interference with proper embalming. It must be remembered that practically all bodies of military personnel have to be shipped great distances and that thorough embalming and preparation of the body are of much more importance than in civilian practice.

It is usually the responsibility of the Laboratory Officer to make the final inspection of bodies. This is a great responsibility and inspection must be thorough to insure that the preparation of the body will assure its arrival at its destination in a wholly satisfactory condition and that the terms of the contract as to clothing, casket and shipping case are fulfilled.

VI. Embalming.—The embalming of the head is readily done by the undertaker when the chest is open, but in his absence may be done very easily by anyone else. If shaving is necessary it must be done before the face is embalmed. The undertaker's press are bottle with several tubes armed with long metal cannula, which are tied into the carotids and subclavian arteries, is most convenient. Pressure is obtained with a pump. If this is not available an alpha eumma syringe will suffice. The tube is tied into the upper thoracic aorta. Of course, the open end of the aorta as well as any leaking arteries (internal hemorrhages) must be closed with clamps or tied.

Undertaker's embalming fluid or a 10 per cent solution of formalin in water, to which a few drops of eosin solution are added to give it the faintest possible tinge of pink, may be used. As the fluid is pumped into the arteries and begins to drive blood before it out of the veins, the face and ears must be massaged and moulded with a gauze sponge into a natural pose, with eyelids and lips closed. The hands should also be massaged until white. When the tissue becomes blanched and firm the process is complete. The same process is applied to the legs, the fluid being injected through the femoral arteries. Some formalin should be allowed to stand for a time in the body cavity. It is well to soak the organs in a 10 per cent formalin solution for several hours before replacing them in the body, making incisions in the solid organs and numerous punctures in the gastrointestinal tract if they have not been opened. Undertaker's hardening compound, oakum or cotton should be spread over the organs after they have been replaced.

VII. Autopsies on Embalmed Bodies.—Under some circumstances, as in the tropics, or when a body is to be shipped some distance in a warm climate, embalming may best be done first and the body then autopsied. Any specimens such as those required for blood cultures, blood chemistry, urinalysis, feces cultures, body fluids, etc., can be obtained before embalming. Cerebral hemorrhage may be seen extremely well in an embalmed body. If the neck organs are to be removed embalming is best performed first. A delay of several hours between embalming and autopsy, or overnight, is perhaps advisable. By this system autopsies may at times be obtained which would be lost otherwise. The properly performed autopsy does not interfere with satisfactory embalming. In fact more thorough embalming can be accomplished after evisceration than by the usual practice of a single-vessel injection and trocharing of the chest and abdomen.

Normal Weights and Measurements

The size and weight of each of the organs varies according to age, stature, and body weight, but the following figures represent an average normal for adult men between twenty and forty years of age.

<i>Brain</i>	
Sagittal diameter	16 to 17 cm.
Vertical diameter	12 to 13 cm.
Weight	1400 gm.
<i>Spinal Cord</i>	
Length	45 cm.
Weight	27 to 28 gm.
Frontal diameters:	
Cervical	13 to 14 mm.
Thoracic	10 mm.
Lumbar	12 mm.
Sagittal diameters:	
Cervical	9 mm.
Thoracic	8 mm.
Lumbar	9 mm.
<i>Pituitary</i>	
Weight	610 mg.

Heart

Weight	3.0 gm.
“	1 to 2 mm.
“	8 to 10 mm.
“	2 to 3 mm.
“	10 cm.
“	7.5 cm.
“	8.5 cm.
“	12 cm.

Pulmonary Artery

Circumference	8 cm.
-------------------------	-------

Aorta

“	7.5 cm.
“	4.5 to 6.0 cm.
“	3.5 to 4.5 cm.

Thyroid

(The size and weight of the thyroid depends upon geographical location, but the normal thyroid should not exceed 40 gm. in weight.)

Measurements	1.5-2.5 x 3-4 x 5-7 cm.
------------------------	-------------------------

Lungs

Weight of right lung	300 to 370 gm.
Weight of left lung	325 to 480 gm.

Liver

Weight	1500 to 1800 gm.
Measurements	25-30 x 19-21 x 6-9 cm.

Spleen

Weight	150 to 200 gm.
Measurements	3-4 x 8-9 x 12-14 cm.

Pancreas

Weight	90 to 120 gm.
Measurements	3.8 x 4.5 x 23 cm.

Kidneys

Weight of each	150 gm.
Measurements	3-4 x 5-6 x 11-12 cm.

Seminal Vesicles

Measurements	0.9 x 1.6-1.8 x 1.1-1.5 cm.
------------------------	-----------------------------

Testis and Epididymus

Weight together	20 to 27 gm.
Measurements of testis	2.2-7 x 2.5-7.5 x 4-5 cm.

Prostate

Weight	15 gm.
Measurements	2.7 x 3.6 x 1.9 cm.

Uterus

Weight of each	5 to 6 gm.
Measurements	0.5 x 2.5 x 3.5 x 5 cm.

Thymus

Weight—21 to 25 years of age	25 gm.
Weight—26 to 35 years of age	20 gm.
Weight—36 to 45 years of age	16 gm.

Gastro-intestinal Tract

.	25 cm.
.	25 to 30 cm.
.	30 cm.
Length of small intestine	550 to 600 cm.
Length of large intestine	150 to 170 cm.

TABLE 91.—LENGTH AND WEIGHT OF FETUS

Time in months	Weight in grams	Length in centimeters
2	4	2 5 to 3
3	5 to 20	7 0 to 9
4	120	10 0 to 17
5	284	18 0 to 27
6	434	28 0 to 34
7	1218	35 0 to 38
8	1549	39 0 to 41
9	1971	42 0 to 44
10	2334	45 0 to 47

Age of fetus in months can be determined after the fifth month by dividing the length in centimeters by 5.

CHAPTER XL

MUSEUM SPECIMENS

By JAMES E. ASH

I. Formulæ for Preservative Solutions.

Kaiserling Solution No. I

Formalin	400 cc.
Water	2000 cc.
Potassium nitrate	30 gm.
Potassium acetate	60 gm.

Kaiserling Solution No. II

Alcohol	80 per cent
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Kaiserling Solution No. III

Potassium acetate	200 gm.
Glycerol	400 cc.
Sodium arsenate	100 gm.
Water	2000 cc.

NOTE.—If sodium arsenate is not available some crystals of thymol, menthol or sodium salicylate may be used instead.

II. Preservation.—To preserve color in gross specimens, they should be quickly washed in water to remove excess blood and placed in Kaiserling's Solution No. I. It is necessary to arrange the specimens in this solution as it is intended for them to appear when finally mounted, as they will become fixed as placed in the solution and it is difficult to alter them after this fixation. The length of time in the No. I solution varies from one to

The specimen should be supported by cotton or suspended by strings so that it will not be in contact with the container. Avoid direct light during all steps.

After fixation, the specimen is rinsed in water, and placed in 80 per cent alcohol (Kaiserling's Solution No. II). When the maximum color has returned, which it will do in a few minutes or an hour or so, the specimen is to be removed from the alcohol, thoroughly washed and then preserved in Kaiserling's No. III. It is necessary to watch the development of the color, for after it has reached a certain point it will begin to fade and it is

color in formalin-fixed tissue, but it is not satisfactory.

III. Preparation.—The preparation of museum specimens must be left largely to the ingenuity of the operator and only a few general principles can be given. The surface to be displayed should represent as large a

section as possible of the whole organ and both the exterior and interior of the organ should be shown. In the case of solid organs such as the liver, a thick slice (5 cm.) should be preserved, as it is impossible to fix a whole liver properly. The thickness of the slab should allow for the removal of a thin layer at a later date to freshen the surface. This is particularly true of the lung, in which case one-half or even the whole organ may be preserved. Nothing solid should be allowed to touch the surface of the fresh tissue until it is fixed and hardened.

It is injurious to pack cotton into a cavity, since after fixation the lining of the cavity will appear merely as a mould of the cotton. If a hollow organ must be held open it is best to distend it with fixing fluid for a day or two before cutting into it. If this is no longer possible, and it must be propped open with cotton, this should at least be inserted very loosely.

The heart after being opened should be stretched on a glass frame in such a way as to display to advantage the chief lesion, or it may be put together and held in its original form during fixation by a few temporary stitches.

time can be filled with Kaiserling
at the ends until hardened, after

be opened, pinned on a board with thumb tacks, so that the mucosa is exposed, and floated face down on the surface of the fixing fluid.

In the case of the kidney, one-half of the organ cleanly cut, forms a satisfactory specimen.

The brain and cord are preserved intact in formalin.

The most satisfactory museum specimen is one which has been intelligently dissected and then fixed. For pure display a rather thick slab of an organ, if cut in the right plane may be better than a whole organ. Numerous incisions in conflicting directions defeat this object, and for that reason it is suggested that at least until a slice or one-half of the organ has been reserved as a possible museum specimen the incisions be bold and slashing to produce smooth parallel surfaces.

CHAPTER XLI

MICROSCOPIC STUDY OF TISSUES

By JAMES E. ASH

I. Formulæ.

Carnoy's Acetic Alcohol Fixative

Glacial acetic acid	10 cc.
Absolute alcohol	60 cc.
Chloroform	30 cc.

Must be freshly prepared. Fixes rapidly and tissues should not remain in it more than three hours.

Mayer's Albumin

White of egg	50 cc.
Glycerol	50 cc.
Sodium salicylate	1 gm.

Shake well together and filter into clean bottle.

Acid-Alcohol

One per cent of hydrochloric acid in 70 per cent ethyl alcohol.

Kinyoun's Carbol-Fuchsin

Basic fuchsin (rosaniline hydrochloride) . .	4 gm.
Phenol crystals	8 gm.
Alcohol, 95 per cent	20 cc.
Water	100 cc.

Decalcifying Fluid

Ten per cent nitric acid in 10 per cent formol-saline, or the following mixture:

Formic acid	50 cc.
Formalin, 10 per cent	50 cc.

Eosin

Eosin Y (di-sodium tetrabromfluorescein) .	0.5 gm.
Alcohol	25.0 cc.
Distilled water	75.0 cc.

This solution will keep indefinitely.

Van Gieson's Mixture

Saturated aqueous picric acid	19 parts
1 per cent aqueous acid fuchsin (di-sodium salt of sulfonated ro-aniline)	1 part

Harris' Hematoxylin

Hematoxylin	1 gm.
Alcohol	10 cc.
(Dissolve dye in alcohol)	
Alum (ammonium or potassium)	20 gm.
Distilled water	200 cc.

The alum is dissolved in water with the aid of heat, and then the alcoholic solution of the dye is added. The mixture is brought to a boil rapidly and then 0.5 gm. of mercuric oxide (red oxide) added. The solution at once assumes a dark purple color and as soon as this occurs the solution is cooled by plunging the flask into cold water. For use, 4 per cent glacial acetic acid is added to the mixture, as this increases the precision of nuclear staining.

Mallory's Phosphotungstic Acid Hematoxylin

Water	100.0 cc.
Hematoxylin	0.1 gm.
Phosphotungstic acid crystals	2.0 gm.

Dissolve the hematoxylin in part of the water with the aid of heat and after it cools add it to the acid dissolved in the rest of the water. Ripen by the addition of 10 cc. of a freshly prepared 0.25 per cent aqueous solution of potassium permanganate.

Weigert's Iron Hematoxylin

30 per cent aqueous ferric chloride	1.00 cc.
Strong hydrochloric acid	0.25 cc.
Distilled water to make	25.00 cc.
1 per cent alcoholic solution hematoxylin	50.00 cc.

This mixture will keep satisfactorily for a week or ten days in a Coplin jar.

Oil Red O (Sudan III or Sudan IV) Fat Stain

Oil red O	1 gm.
Acetone	50 cc.
70 per cent alcohol	50 cc.

Zenker's Fluid

Potassium bichromate	2 gm.
Bichloride of mercury	5 gm.
Water	100 cc.

Just before use add 5 cc. glacial acetic acid to 95 cc. of the above for acetic-Zenker. If formal-Zenker is desired, use 10 cc. of formalin with 90 cc. of the above solution, the formalin taking the place of the acetic acid.

Carbol-xylene

Phenol	1 part
Xylene	3 parts

II. **Frozen Section Method.**—It is possible with this method to prepare a slide for examination in a few minutes so that it is particularly applicable to "operating-room diagnosis." It is also the method of choice when it is desired to stain for fat.

1. Materials.

Automatic freezing microtome, and sharp knife.
 Tank of CO₂ and connections; the tank to be mounted inverted or on its side.
 A shallow dish filled with water.
 A mounted needle, glass rod drawn to dull point, or pair of fine, smooth-pointed forceps.
 2 pyrex test tubes.
 Bottle of 10 per cent formalin.
 Bottle of 1 per cent ammonia water.
 Bunsen burner or alcohol lamp.
 Glass slides and cover glasses.
 8 small glass dishes.
 Harris' hematoxylin.
 Eosin.
 95 per cent alcohol.
 Absolute alcohol.
 Carbol-xylene.
 Xylene.
 Canada balsam.
 Blotting paper.

2. Technic.—If the tissue has not already been fixed, a block about 0.5 cm. thick is boiled for one minute in 10 per cent formalin in a test tube, and rinsed in tap water. It is placed on the freezing stage of the

Select full sections and transfer in turn to the following which are in small shallow glass dishes:

1. Harris hematoxylin	30-60 secs.
2. 1 per cent ammonia water	Until blue
3. Tap water	Rinse
4. Eosin	5-15 secs.
5. 95 per cent alcohol	Rinse
6. Absolute alcohol	5 secs.
7. Carbol-xylene	5 secs.
8. Xylene	5 secs.
9. Mount on slide in Canada balsam.	

III. Routine Paraffin Method.—1. Materials.—The following are required in addition to those enumerated under Frozen Section Method:

Chloroform.
 Acid alcohol.
 Xylene.
 12 bottles, wide mouth, 4-ounce with corks.
 Tissue forceps, 1 pair each, long and short.
 Sectioning razor or razor blades.
 1 scalpel.
 Cutting board (cork or paraffin block).
 Paper or metal forms for moulding blocks.
 2 basins or photo-developing trays.
 Ice.
 Rotary microtome.
 Sharp knife.
 Hone and strop.
 Camel's hair brushes, one pointed, one 1 to 1½ inches wide.
 12 Coplin jars.

2. **Fixation.**—Specimens of tissue which are to be examined microscopically should be fixed as quickly as possible after surgical removal or after death of the patient.

A 10 per cent solution of formalin is the most convenient and generally practicable fixative. For finer cellular studies, and some special stains, it is necessary to fix in one of the chromate solutions, of which Zenker's is the most popular. If it is desired to stain for glycogen, aqueous fixatives must be avoided as glycogen is soluble in water, and absolute alcohol must be used.

Blocks of tissue should be about 0.5 cm. in thickness and they should be placed in an excess of the fixative agent to insure thorough impregnation. If formalin is used, the tissue may be kept in it indefinitely. If fixation is in Zenker's, the blocks are to remain in it but twenty-four hours, then washed in running water for twenty-four hours and preserved in 80 per cent alcohol.

3. **Embedding and Cutting.**—Blocks not more than 0.5 cm. thick that have been fixed in 10 per cent formalin or Zenker's fluid are placed in the following:

- (1) Ninety-five per cent alcohol, two to four hours.
- (2) Absolute alcohol, two to four hours.
- (3) Chloroform, two to four hours.
- (4) Chloroform saturated with paraffin, overnight in warm place at about 37° C.

(The above steps are carried out in wide-mouth, tightly corked bottles).

- (5) Paraffin two to four hours in oven.
- (6) Embed in paper or metal forms, with desired surface down, being sure to eliminate air bubbles. To prevent crystallization of paraffin, the mould should be immersed in ice-water while paraffin is still melted.
- (7) Trim block so that opposite edges about tissue are parallel, leaving narrow margin of paraffin.
- (8) Mount on metal block holder by heating latter, pressing on block and immersing all in ice-water.
- (9) Cut sections as thin as possible; under 10 μ . Be sure knife is sharp, tightly clamped in microtome, that its edge is inclined toward block just enough so that block misses back surface of knife and that lower edge of block and knife edge are parallel.
- (10) Lay sections on surface of water sufficiently warm to insure complete spreading of section (40° to 50° C.). If sections are in ribbons, they may be separated by touching while in the water with the edge of a heated scalpel.
- (11) Float section onto slide that has been very lightly smeared with Mayer's albumin.
- (12) Drain off water and place slide in oven for one-half hour to fix albumin.

4. **Staining.**—In the following steps, use a series of Coplin jars lined up in proper order.

Remove paraffin by immersing slide in xylene for several minutes, then in absolute alcohol one minute.

Ninety-five per cent alcohol one minute. (If tissue has been fixed in Zenker's, add sufficient iodine to the alcohol to give it a light port-wine color. This is to remove the precipitate of mercury and requires five to ten minutes. Rinse in clear 95 per cent alcohol.)

Wash in tap water

10 minutes

Wash in tap water.

80 per cent alcohol containing 1 per cent ammonia,
until blue about 10 seconds

Wash in tap water.

Eosin 2 minutes

95 per cent alcohol—to remove excess eosin.

Absolute alcohol 1 minute

Carbol-xylene 1 minute

Xylene—2 changes 2 minutes each

Mount in Canada balsam.

IV. **Rapid Paraffin Methods.**—Two methods are given, either of which is satisfactory.

Ambrogi Method

1. Formalin, 10 per cent 6 hours
(or overnight)
2. Wash in running water 2 hours
3. Acetone through 3 changes of one-half hour each 1½ hours
4. Cedarwood oil until translucent.
5. Paraffin through 3 changes of one hour each 3 hours

Mallory and Wright (Method No. 3)

Tissues already fixed in formalin or fresh tissues boiled two to three minutes in 10 per cent formalin may be used. Blocks should not be more than 5 mm. thick.

1. Acetone, 2 or 3 changes 1-2 hours
2. Benzene, 2 changes 30 minutes
3. Paraffin, 2 changes 45 minutes each

Following either of above methods proceed as in step No. 6 under Routine Paraffin Method.

V. **Special Stains.**

Stains for Fat

1. Stain frozen sections for one minute in oil red O solution.
2. Wash in water
3.
4.
5.
6. Clear in aniline.
7. Mount in glycerol.

The fat is stained red.

Campbell's Stain for Acid Fast Bacilli

After removing paraffin and passage through alcohols and water—

1. Stain sections for one-half hour at room temperature in Kinyon's carbol-fuchsin.
2. Wash in water.
3. Decolorize in acid alcohol a minute or two.
4. Wash in water.
5. Hematoxylin.

6. Wash in water.
7. Acid alcohol.
8. Wash in water.
9. Ammonia water, 1 per cent.
10. Wash in water.
11. Counterstain in a saturated aqueous solution of Orange G.
12. Dehydrate in 95 per cent and absolute alcohol.
13. Clear in xylene.
14. Mount in balsam.

MacCallum's Stain for Bacteria

Paraffin sections affixed to the slide are passed through xylene and alcohols to water, and stained for ten minutes to one-half hour in the Goodpasture mixture prepared as follows:

30 per cent alcohol	100.00 cc.
Basic fuchsin	0.59 gm.
Aniline	1.00 cc.
Phenol crystals	1.00 gm.

They are then washed in water and differentiated in 40 per cent formaldehyde. This requires only a few seconds, the bright red color being washed away and replaced by a clear rose. Sections are next washed in water and counter-stained in a saturated aqueous solution of picric acid. The section remains in this until it assumes a purplish color (three to five minutes); then it is washed in water and differentiated in 95 per cent alcohol. The red reappears and some of it is washed out as is some of the yellow of the picric acid. The sections are then washed in water and stained for three to five minutes in Sterling's gentian violet prepared as follows:

Crystal violet	5 gm.
Alcohol	10 cc.
Aniline	2 cc.
Water	88 cc.

Then washed in water and immersed in Gram's iodine solution one minute.

Iodine	1 gm.
Potassium iodide	2 gm.
Water	300 cc.

The slides are finally blotted dry without washing, treated in equal parts of xylene and aniline until no more color comes away, passed through 2 changes of xylene and mounted in balsam.

Gram-negative organisms are stained red; Gram-positive, blue.

Warthin-Starry Method (Kerr modification).

This method has the advantage over the Levaditi method in that it is used on tissues already embedded in paraffin.

1. Fixation: Formalin. Zenker-fixed is not satisfactory.

2. Solutions:

(a) Citric acid: One per cent aqueous solution. It should be not over one week old.

- (b) Acidulated water.

To 5 liters of doubly distilled water, add enough 1 per cent citric acid to produce a pH of 4.4. The pH can be determined satisfactorily by using bromocresol green as an indicator. Usually 10 drops of citric acid to 100 cc. of doubly distilled water is sufficient.

(c) Silver nitrate solution. Dissolve 4 gm. of reagent quality silver nitrate in 200 cc. of acidulated water. Store in a dark bottle.

(d) Hydroquinone solution. Dissolve 1 gm. of hydroquinone in 33 cc. of acidulated water.

(e) Gelatin solution. Heat 200 cc. of acidulated water to boiling in a 500-cc. beaker. Remove the flame and crowd in 10 gm. of gelatin (bacteriology quality). Stir intermittently.

(f) Developer. Mix 3 cc. of the silver nitrate solution and 1 cc. of hydroquinone solution with 15 cc. of gelatin.

3. Procedure.

(a) Cut sections 8 to 10 microns thick from paraffin-embedded tissue. Float them on warm, recently boiled, freshly distilled water.

(b) Without the use of albumin or other fixative, the sections are placed on No. 1 cover slips which have been cleaned by soaking in concentrated nitric acid, washed in distilled water and dried out of 95 per cent alcohol. The sections on the cover glasses are dried in the oven for as long as twenty-four hours, if time permits.

(c) Warm the cover glass preparations gently and place in 2 changes of xylene to remove paraffin, then in 2 changes of absolute alcohol.

(d) Rinse in 95 per cent and in 70 per cent alcohol, in distilled water and finally in acidulated water.

(e) Place cover slips on edge in 1 per cent silver nitrate solution prepared by diluting the 2 per cent solution with acidulated water and keep in the oven at 55° to 60° C. for thirty minutes.

(f) Place cover slips, section up, in a Petri dish and pour thoroughly mixed developer over them.

(g) Allow the sections to develop in this mixture until they turn a light, golden brown or a grayish yellow, depending upon the kind of tissue. Some tissues will develop more rapidly than others. The developing time for a given tissue can be determined best by treating several sections from the same block for graded intervals, in steps of one-fourth minute, from one minute to three or more minutes. After some practice the technician will learn to recognize by the color of a section when a desirable level of development has been obtained. Underdeveloping gives a very light background with pale or attenuated black spirochetes. Overdeveloping gives a dark background and heavy black spirochetes with the characteristics of the background with

water, then rinse in distilled water at room temperature. (3° to 55°) distilled

(i) Rinse in 70 per cent and in 95 per cent alcohol.

with tap and distilled water, and drying.

4. Results. Spirochetes stain black. Background is yellowish.

*Masson's Trichrome Stain (Lt. Col. DeCoursey)**Solutions:*

1. Weigert's Hematoxylin:

Solution A

Hematoxylin	1.5 gm.
Alcohol, 95 per cent	100 cc.

Solution B

.	4 cc.
.	95 cc.
.	1 cc.

For use mix equal parts of A and B.

2. Ponceau—acid fuchsin mixture (Krall):

Ponceau de xyli-dene (Krall-Eimer and Amend, N. Y.)	0.9 gm. (approx.)
Acid fuchsin	0.1 gm. (approx.)
Glacial acetic acid	1 cc.
Distilled water	100 cc.

3. Phosphomolybdic acid 1 gm.
 Distilled water 100 cc.

4. Aniline Blue:

Boil 100 cc. of distilled water. When boiling remove and add 2 or 3 grams of aniline blue. Boil a little longer to dissolve the dye. Add 2.5 cc. of glacial acetic acid. Cool and filter.

Fixation: (For Masson's Trichrome Stain.)

Bouin best, but any fixative may be used. The paraffin sections having been carried through xylene and alcohols to water, are soaked overnight in Bouin—even Bouin-fixed tissues. Just before staining, wash in running water for twenty minutes, to remove picric acid.

Staining:

1. Quick rinse
differentiate in
2.
3.
4.
5. decolorizes the
6. Leave five or
7.
8. This step is
omit this step
with light green.
9. Rinse with distilled water.
10. Glacial acetic acid, 1 per cent in distilled water (Coplin jar) two to five minutes. This eliminates the phosphomolybdic acid and sets the acid red and blue.
11. Dehydrate: Clear with toluene or xylene.

* After soaking paraffin sections in Bouin's solution overnight.

12. Mount with Canada balsam.

(Ju

oluene

its the

Van Gieson's Stain for Connective Tissue

Either formalin or Zenker-fixed tissue may be used. Paraffin sections are brought through xylene and the alcohols to water, then

1. Stain in Weigert iron hematoxylin, five to ten minutes.
2. Wash in water.
3. " " " " " "
4. " " " " " "
5. " " " " " "
6. Cedarwood oil, one minute.
7. Xylene and mount in xylene balsam.

Nuclei are stained black or dark brown; connective tissue a purplish-red; stroma yellow.

Mallory's Phosphotungstic Acid Hematoxylin Stain

Zenker-fixed tissue gives the best results and only if tissue is fresh. If formalin-fixed, wash in water and mordant for twelve to twenty-four hours at 37° C. in Zenker's fluid without the acetic acid. Paraffin sections fixed to slides are brought through xylene and the alcohols to water.

1. Then place in 0.25 per cent aqueous solution of potassium permanganate for five to ten minutes.
2. Wash in water.
3. " "
4. " "
5. " "
6. " " (This dehydration must be done quickly because alcohol readily extracts the red part of the stain.)
7. Xylene and balsam.

Nuclei, centrosomes, achromatic spindles and fibroglia, myoglia and neuroglia fibrils, fibrin and the contractile elements of striated muscle are d the ground substances to brownish-red. Coarse

Wilder's Reticulum Stain

Fixation.—Fix tissues in 10 per cent formalin, acetic-Zenker or formol-Zenker.

xylene, graded alcohols and distilled water before staining. Celloidin sections may vary in thickness from 4 to 30 microns. The thick sections give a better idea of the density of the fibers in some tumors. They are stained in dishes before mounting. Frozen sections may be stained in dishes before mounting, or mounted on slides and attached with thin celloidin before staining.

Pretreatment.—Place paraffin sections in 10 per cent phosphomolybdic acid, or celloidin and frozen sections in 0.25 per cent potassium permanganate, for one minute. Rinse in distilled water. Place celloidin and frozen sections in hydrobromic acid (Merck's concentrated, 34 per cent, 1 part; distilled water, 3 parts) for one minute. Hydrobromic acid may be omitted following the use of phosphomolybdic acid, in staining paraffin sections.

Sensitization.—Wash in tap water, then in distilled water and dip in 1 per cent uranium nitrate (sodium free) for 5 seconds or less.

Impregnation.—Wash in distilled water ten to twenty seconds and place in silver diamino hydroxide (Foot) for one minute:

To 5 cc. of 10.2 per cent silver nitrate add ammonium hydroxide drop by drop until the precipitate which forms is dissolved. Add 5 cc. of 3.1 per cent sodium hydroxide and just dissolve the resulting precipitate with a few drops of ammonium hydroxide. Make the solution up to 50 cc. with distilled water.

Reduction.—Dip quickly in 95 per cent alcohol and reduce for one minute in the following solution:

Distilled water, 50 cc.; 40 per cent neutral formalin (neutralized with magnesium carbonate), 0.5 cc.; 1 per cent uranium nitrate, 1.5 cc.

Toning.—Wash in distilled water and place in 1:500 gold chloride (Merck's reagent) one minute. Rinse in distilled water. Place in 5 per cent sodium thiosulphate (hyposulphite) one to two minutes.

Counterstaining and Mounting.—Wash in tap water; counterstain, if desired, with hematoxylin and Van Gieson, or hematoxylin and eosin; dehydrate in alcohol. Clear in xylene and mount in balsam. The use of ammonia must be avoided in blueing sections after hematoxylin as it dissolves the silver.

The use of distilled water and clean glassware for all solutions is essential. All the solutions may be used repeatedly and kept in Coplin jars for several days. The solutions keep for an indefinite time in amber glass-stoppered bottles without disintegrating.

Stain for Elastic Tissue

Sections fixed to the slide are brought through xylene and alcohols to water, and stained in the following for one-half to two hours.

Crystal violet	2.0 gm.
Dextrin	0.5 gm.
Resorcinol	4.0 gm.
Water, distilled	200.0 cc.

This mixture is boiled in a flask and when boiling briskly 25 cc. of 29 per cent aqueous solution of ferric chloride are added. Boiling is continued for two to five minutes. A heavy precipitate forms, and the mixture assumes a greenish cast. It is then cooled and filtered. The precipitate is dissolved by boiling with 200 cc. of 95 per cent alcohol over a water bath or electric hot plate. After cooling and filtering the volume is made up to 200 cc. with 95 per cent alcohol. Add 4 cc. of strong hydrochloric acid (33.8 per cent).

Sections are differentiated in 95 per cent alcohol, washed in water and stained for ten to fifteen minutes in Weigert iron hematoxylin.

Sections are washed in water and stained in Van Gieson's mixture for one to two minutes.

Sections are then differentiated in 95 per cent alcohol, dehydrated through alcohols, cleared in xylene and mounted in balsam. Elastin only stains yellowish-green.

Campbell's Stain for Nerve Tissue

The following impregnation method for medullated and non-medullated nerve fibers has the advantage of allowing a counterstain for the differentiation of other tissue elements. It has been found satisfactory for staining the fibers of both the central nervous system and peripheral nerves.

1. Fix pieces of tissue not over 3 mm. thick for three hours in Carnoy's fluid.
2. Wash in several changes of absolute alcohol for twenty-four hours.
3. 50 per cent alcohol for six hours.
4. Ammoniated 50 per cent alcohol (5 drops of ammonium hydrate to 50 cc. of 50 per cent alcohol) for twenty-four hours.
5. Rinse quickly in distilled water.
- 6
- 7
8. Hydroquinone 1 gm.
9. We
10. Place in 80 per cent alcohol.

Steps 6, 7 and 8 should be carried out in the dark at 37° C. and Step 9 in the dark at room temperature. The Carnoy's fluid, silver solution and reducing solution should be freshly made up. It is essential to have clean glassware and to use distilled water throughout.

A satisfactory impregnation of the nerve fibers, but with less sharp differentiation, may be obtained by placing formalin-fixed blocks in Carnoy's solution for twelve to twenty-four hours and then proceeding as above.

After the tissue has been dehydrated, embedded and cut according to the usual paraffin technic it may be counterstained with hematoxylin and eosin, and mounted in balsam.

Nerve cells stain brown, their nuclei blue and their processes black. Non-medullated fibers stain intensely black and medullated fibers brownish-black. The medullary sheath appears as a yellow segmented cylinder surrounding its darker axon. Where a neurilemma is present its oval blue nuclei are easily distinguishable. Elastic tissue stain white fibrous connective tissue pink the age of the fibers. Muscle stains yellow and all nuclei blue.

Stain for Nerve Cells

Paraffin sections are brought through xylene and the alcohols to water and then stained in the following:

Azure C (monomethyl thionin)	0.25 gm.
Erythrosine	0.10 gm.
Water	95.00 cc.
95 per cent alcohol	5.00 cc.

Dissolve the azure in 50 cc. water.

Dissolve the erythrosine in 5 cc. of 95 per cent alcohol and add 45 cc. water.

The 2 solutions are mixed and filtered. The stain should be filtered each time before using, and it will sometimes be necessary to add a small amount of erythrosine to it as it gets older.

Best results are obtained by leaving sections from central nervous system in stain overnight; for other tissues four hours are usually sufficient.

For formalin-fixed material the differentiation is as follows:

1. Excess of blue is removed with absolute methyl alcohol in ten seconds.
2. Differentiate in absolute ethyl alcohol, 50 cc.; glacial acetic acid, 10 drops; watch process under microscope until the background is pale pink and the nerve cells a clear blue.
3. Wash thoroughly with absolute alcohol.
4. Clear in oil of bergamot.
5. Xylene.
6. Mount in neutral balsam or thick cedar oil.

For alcohol-fixed material differentiate in:

1. 0.5 per cent aluminum sulfate.
2. 50 per cent alcohol.
3. 80 per cent alcohol.
4. 95 per cent alcohol.
5. Absolute alcohol.
6. Oil of bergamot.
7. Xylene.

Caution.—The absolute alcohol used in differentiation must be high grade, practically an anhydrous alcohol—or the blue stain will be extracted.

Stain for Amyloid

Paraffin sections are brought through xylene and the alcohols to water then,

1. Stain in 1 per cent aqueous solution methyl violet, five minutes.
2. Wash in water containing 1 per cent glacial acetic acid.
3. Mount in water or glycerol.

The stain is not permanent. Amyloid stains violet-red, the tissue blue.

Stain for Hemosiderin

Paraffin sections of freshly fixed tissue stained for about one-half minute in—

1.
2. Wash thoroughly in several changes of water.
3. Counterstain in 0.1 to 0.5 per cent solution of basic fuchsin in 50 per cent alcohol for five to twenty minutes.
4. Wash in water.
5. 95 per cent alcohol few minutes.
6. Absolute alcohol few minutes.
7. Xylene and mount in balsam.

Nuclei and hemofuscin are stained bright red, hemosiderin blue.

Von Kossa's Stain for Calcium

1. Climate.
2. 1 a 1 to 5 per cent solution
3. 2 twelve hours.
4. ~~in Canada balsam.~~

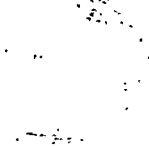
Calcium stains deep black; nuclei may be counterstained with alum hemotoxylin, carmine or safranin.

VI. Decalcification.—Tissues containing bone are fixed in 10 per cent formalin, then placed in the decalcifying fluid until the lime salts are removed, changing fluid daily. This requires from one to seven days and may be determined by piercing the block with a needle. Suspend the tissues in neutral 10 per cent formalin over magnesium carbonate until blue litmus paper is not changed by touching the tissue to it. Then wash in running water for twenty-four hours, after which the blocks are ready for dehydration and embedding, beginning with step 1 of Routine Paraffin Method.

For bone marrow studies on very thin slices of bone or sternal-puncture material use Zenker's fixative with 10 per cent acetic acid instead of the usual 5 per cent. Twenty-four to forty-eight hours are required, then proceed as after the regular fixation. The best stain, incidentally, is Mallory's Phloxine and Methylene Blue.

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PART X

Special Veterinary Laboratory Methods

By **RAYMOND RANDALL**

IN addition to many of the methods which have already been considered in this Manual, the following additional technical procedures are also commonly employed in the veterinary diagnostic laboratory.

CHAPTER XLII

GENERAL TECHNIC

1. Collection of Specimens.—**1. Serum.**—Blood is usually collected from the jugular vein of the horse, sheep or cow, while from the dog it may be drawn from the saphenous vein. Specimens of serum should be clear, uncontaminated and free from hemoglobin.

2. Blood.—For bacteriological examination and smears on slides, treat in the same manner as specimens from man. For examination for parasites, blood is collected in physiological saline containing 1 per cent sodium citrate. For anthrax, the ear of a dead animal may be severed close to the skull, the exuding blood collected with a sterile pipet or syringe, distributed over the inside wall of a test tube or bottle and permitted to dry before stoppering. For blood cell counts the technic is the same as described for man, except that for the white cell count 1 to 1.5 per cent acetic acid solution is used for the diluting fluid since the 0.5 per cent solution used for human blood may not cause complete laking of the red blood cells. The normal blood cell counts of domestic animals are shown in Table 92.

TABLE 92.—NORMAL BLOOD COUNTS

	Species				
	Horse	Cow	Pig	Dog	Cat
Hemoglobin (%)	70-85	60-75	75-85	85-90	65-75
Red blood cells (millions)	6-9	5-7	6-8	5-7	5-6
White blood cells (thousands)	6-9	5-8	15-20	7-10	10-12
Polymorphonuclears (%)	60-65	40-45	38-42	67-70	58-60
Small lymphocytes (%)	25-30	48-52	52-54	25-28	35-38
Large lymphocytes (%)	2-5	4-6	4-5	3-4	2-4
Transitionals (%)	1-2	2-4	1-3	1-2	2-3
Eosinophiles (%)	3-4	5-7	4-5	4-6	4-5
Mast cells (%)	0 5-1 0	0 5-1 0	1 0-2 0	0-0 5	0-0 6

3. Tissue.—Tissues for histopathological examination should be prepared as indicated in the section on Pathology. Tissues for bacteriological examination may be sprinkled with powdered borax, wrapped in sterile animals It is tory is distant, the brain should be removed, one hemisphere placed in pure

glycerol for touch preparations and animal inoculation; and the remainder placed in 10 per cent formalin for histopathological examination. For equine encephalomyelitis, portions of brain may be preserved for shipment in buffered (pH 7.4 to 7.5) 50 per cent glycerol in physiological saline.

4. **Skin Scrapings.**—Specimens to be examined for fungi or external parasites should be collected from the periphery of the most recent lesions. Use a sharp scalpel and take the scrapings deep enough to insure inclusion of some hair roots.

5. **Urine.**—Specimens of urine, especially those for bacteriological examination, should be collected with a sterile catheter after disinfection of the external genitals. Specimens for chemical examination may be preserved for shipment by using 2 drops of formaldehyde for each ounce of urine.

6. **Feces.**—Specimens should be obtained from freshly expelled material, and collected in a manner to exclude portions that have come in contact with the ground. For microscopic examination for ova, it is always advisable to concentrate the specimens. Prepare and keep on hand a 75 per cent aqueous solution of ordinary cane sugar, adding benzoic acid as a preservative. Place several grams of the feces in a clean beaker and mix thoroughly with a glass rod with sufficient water to make a uniform suspension. Filter through gauze to remove coarse particles, add an equal amount of sugar solution and mix. Place in centrifuge tube and centrifugalize at a moderate rate of speed for about two minutes. By touching the butt of a small test tube or the rounded end of a glass rod to the surface of the fluid in the centrifuge tube a few drops of the uppermost layer may be picked up, placed on a slide and the preparation examined microscopically for ova. The specific gravity of the sugar solution is such that the ova of most parasites are brought to the top.

7. **Forage and Bedding.**—Specimens for bacteriological examination should be representative of the total lot.

8. **Poisonous Plants.**—Specimens for examination or identification should, when practicable, include the root, flowers and seeds. If expert opinion is desired, the specimens may be sent to the Pathological Division, Bureau of Animal Industry, United States Department of Agriculture, Washington, D. C., or to the nearest College of Agriculture.

CHAPTER XLIII

DIAGNOSIS OF SPECIFIC DISEASES

I. Actinomycosis.—Caused by: *Actinomyces bovis*.—This infection occurs commonly in cattle and hogs, infrequently in horses and sheep. In tissue processes it forms minute, grayish, yellowish, or greenish granules which, when crushed and examined microscopically, are found to be made up of a dense network of branching threads of filamentous mycelia radiating from a central point and terminating at the periphery in characteristic club-shaped endings.

In making a direct microscopic examination, mix a portion of pus or exudate with a quantity of water or salt solution and with the aid of a hand lens examine for granules. If present they are placed on a slide, crushed, and stained with carbolfuchsin. The "ray fungus" is characteristic. Histopathological sections of tissues from cases of actinomycosis reveal the organism satisfactorily when stained with hematoxylin-eosin.

The mycelial filaments are uniformly Gram-positive, whereas the club-shaped terminals are usually Gram-negative. This assists in differentiating the granules of *Actinomyces bovis* from *Actinobacillus lignieresii*. It is extremely difficult to isolate in pure culture. For cultural and biochemical characteristics, see page 498.

II. Gangrenous Dermatitis and Other Necrobacilloses.—Caused by: *Actinomyces necrophorus*.—In addition to gangrenous dermatitis in equines, this organism is the etiological agent of a variety of conditions collectively referred to as "necro-bacilloses." These include foot-rot and lip-and-leg ulceration of sheep, necrotic stomatitis of swine, calf diphtheria, multiple liver abscesses in cattle, sheep and hogs, and a number of localized processes. It occurs secondarily in intestinal ulcers in hog cholera and in canker of the foot in the horse. It has been isolated from necrotic lesions in man. Pure cultures may be obtained from abscesses in the liver upon media containing blood or blood serum. Of the anaerobic methods, the hydrogen replacement method is least suitable. The alkaline pyrogallate method of oxygen exhaustion is fairly satisfactory.

III. Anthrax.—Caused by: *Bacillus anthracis*.—If dried blood is examined, several cc. of sterile physiological salt solution are placed in the vial or tube containing the same and, with a platinum loop the blood is washed from the walls of the container, thoroughly mixed, and used for inoculation of culture media as well as laboratory test animals. If an ear is submitted, blood from the auricular veins may be moistened with physiological salt solution and treated as above.

Streaked cultures are made on agar plates and incubated for twenty-four hours, and preparations from characteristic colonies are examined for morphology and motility. Suspected organisms should be tested for

motile and, later, spore-forming, aerobic organisms, a diagnosis of anthrax is justified.

Cultural characteristics may be found in Part IV, Bacteriology.

IV. Blackleg (also called Symptomatic Anthrax).—Caused by: *Clostridium chauvæi*.—When freshly isolated from tissues (in contradistinction to anthrax) the organism shows the presence of spores. Intramuscular inoculation into the hind leg of a guinea pig of organisms or extracts of comminuted tissue causes death in eighteen to thirty-six hours. The lesion, emphysematous gangrene, gives off an odor resembling that of butyric acid. The sporulating organisms may be demonstrated by direct smear and are usually single or in pairs. Rabbits and rats are not susceptible.

See Part IV, Bacteriology, for cultural and differential characteristics of the Clostridia.

V. Malignant Edema.—Caused by: *Clostridium septicum* (also called *Cl. adematidis-maligni*, *Vibrio septique*).—This organism is constantly present in soil the world over. It usually enters the body through wounds, and as a rule the disease in animals terminates fatally. It causes one type of gas gangrene in man. It is a strict anaerobe and may be cultivated by using the same technic as described for *Cl. chauvæi*. Guinea pigs, rabbits, chickens, pigeons and mice are susceptible but the rabbit is preferable, as it differentiates malignant edema from blackleg to which the rabbit is refractory.

VI. Glanders.—Caused by: *Malleomyces mallei*.—Lesions in subcutaneous and other lymph glands, nodules from the lungs and elsewhere, as well as pus from suspected lesions, should be subjected to direct microscopic examination, culture, histopathological examination and animal inoculation. Serum specimens should be examined by the complement-fixation test.

Tissues should be seared to destroy external contaminants, and representative portions removed aseptically, comminuted with sand in a sterile mortar, and planted on 3 per cent glycerol-agar potato medium, or in 3 per cent glycerol bouillon. Media of a slightly acid reaction (pH 6.8) are best. As *M. mallei*, when freshly isolated, grows slowly, it may require several days or one week for colonies to become visible. See Part IV, Bacteriology, for cultural characteristics.

Histopathological preparations may show the typical glanders tubercle. Care must be taken not to mistake small parasitic nodules for those of glanders. The glanders tubercle is, in some respects, similar to the lesion of tuberculosis. Usually there is a caseous center surrounded by an area of cellular infiltration which becomes less dense as it extends toward the periphery of the lesion. There is more or less fibrous tissue present depending upon the age of the process. Giant cells and deep-staining chromatin granules (derived from nuclei of broken down cells) are observed. Parasitic nodules should be readily differentiated by the fact that the lesion is usually sharply circumscribed and encapsulated, calcification is the rule in all but very young lesions and, finally, there is almost invariably a marked eosinophilia.

When grown on either of these media, especially when the surface is

somewhat dry, the growth, in seventy-two hours, takes on a characteristic dry, wrinkled appearance like parchment. On plates it will be found that the colonies are non-adherent to the medium and can be removed without material distortion, by pushing them lightly with a platinum loop. Old cultures may lose their parchment-like appearance found on page 471. *Streptococcus genit.* is but weakly Gram-positive.

This reaction may prove of value where the animal has not received abortion bacterin, but the test may not be positive even in actual cases if the blood is collected within the period from a few days before to a week after abortion.

For charge is best collected by the use of sterile swabs. Small sections of umbilical cord, fetal spleen, liver, ligated heart, and kidney may be examined. The fetal stomach with fluid contents should be ligated and submitted for examination.

VIII. Infectious Abortion of Cattle.—Caused by: *Brucella abortus* (Bang) in a large per cent of cases; rarely *Vibrio fetus*. The organism commonly causing abortion in cattle is one of three closely related types, *Brucella abortus*, *Brucella suis*, and *Brucella melitensis*. The differentiation of these three varieties is not a simple matter and is described in Part IV, Bacteriology. Serum specimens from cows suspected of having the disease may be used in the agglutination test.

Guinea pig inoculation will frequently result in isolation of *Brucella abortus* when direct artificial culture methods fail. If the material to be inoculated is not too grossly contaminated the guinea pig may be inoculated intraperitoneally, otherwise the inoculation should be made subcutaneously. Six weeks after inoculation the guinea pig is chloroformed and the spleen, liver, testicles, and the inguinal, sublumbar and precrucial lymph glands are examined for lesions. The spleen is the best organ for culture and generous amounts of the same should be rubbed over liver infusion agar. *Brucella abortus* when freshly isolated will grow only in an atmosphere of reduced oxygen tension such as 10 per cent carbon dioxide.

If *Vibrio fetus* is suspected, the uterine exudate and material from the chorion and fetal tissues, especially the stomach and kidneys, should be examined microscopically and the organism is significant.
an atmosphere of reduced oxygen

Media containing blood or serum are essential to support growth.

IX. Hemorrhagic Septicemia.—Caused by: *Pasteurella bollingeri* (in cattle); *Pasteurella equiseptica* (in horses); *Pasteurella suilla* (in swine); *Pasteurella oriseptica* (in sheep); and *Pasteurella oricula* (in birds). (See Part IV, Bacteriology, for cultural characteristics.)

Blood cultures, blood smears on slides, tissue and tissue exudates from edematous swellings, affected lymph glands, spleen and other organs should be examined.

Blood agar or other solid media containing serum are to be preferred and it is always well to include E.M.B. agar for the detection of organisms of the coli group as these latter, at times, play a pathogenic rôle and when present in tissues may show bipolar staining. Stained preparations from

young bouillon cultures may show bipolar staining if the stain is not left on too long.

For microscopic demonstration of the organism, blood films, as well as preparations from the spleen and kidneys, are best stained with Wright's stain. Dilute carbolfuchsin and Loeffler's methylene blue give fair results.

Specimens from suspected cases of hemorrhagic septicemia may also be inoculated into rabbits and mice, into the former intravenously and the latter intraperitoneally. If the material is grossly contaminated the inoculations may be made subcutaneously. For the recovery of the organism from such animals, cultures should be made from the heart blood, spleen, kidneys, peritoneal fluid and any prominent lymph glands.

X. Ulcerative Lymphangitis.—Caused by: *Corynebacterium oris* (commonly called Preisz-Nocard bacillus).—Lymph glands, lung, spleen and other tissues showing lesions as well as pus should be examined for the specific organism either by direct microscopic examination or isolation on neutral agar. Morphologically, the organism is indistinguishable from some strains of *C. diphtheriæ*. Biochemical characteristics are given in Part IV, Bacteriology. Subcutaneous and intraperitoneal inoculations of male guinea pigs with pus and tissue extracts produce metastatic abscesses and, in many instances, an orchitis. Rabbits infected by intravenous injection become emaciated and die after several weeks. Caseous foci from which the organisms may be isolated are usually found in the liver, lungs and lymph glands.

also
be made. However, animals infected with this organism do not give reactions to the mallein test, nor do their sera give a reaction to the complement-fixation test for glanders.

XI. Epizootic Lymphangitis.—Caused by: a fungus, *Blastomyces farciminosus* (*Zymonema farciminosum*).—This organism is readily demonstrable in pus either stained or unstained from affected lymphatics. Dilute carbolfuchsin, gently heated, gives good staining results, as does the Gram's method. With the latter stain the blastomyces retain the gentian violet; however, if the decolorization is continued, the organisms frequently appear completely Gram-negative. They are doubly contoured oval cells 3 to 4 μ in length and 2.5 to 3.5 μ in width. Occasionally budding processes may be observed. Cultivation on artificial media is rarely successful on initial isolation and when growth does occur it is imperceptible until after twenty to forty days. Egg medium or Sabouraud's agar is preferred. Guinea pigs inoculated in the inguinal region sometimes develop lesions in the regional lymph glands and rabbits may develop lesions at the point of inoculation.

XII. Mange.—Caused by: Small mites belonging to the order Acarina. Occurs in practically all species of animals. These mites are easily detected on microscopic examination by their size and shape.

XIII. Ringworm.—Examine the hair roots and follicles for the presence of *Trichophyton*, a fungus which forms chains of segments. (See Part III, Mycology.)

XIV. Trypanosomiasis.—Caused by: *Trypanosoma equiperdum* (Dourine); *Trypanosoma evansi* (Surra); *T. brucei* (Nagana); *T. hippicum* (Murrina).—It is difficult to demonstrate the trypanosome of dourine in the blood or fluid of the edematous plaques of horses; however, they may

be found in the peripheral circulation in cases of surra, nagana and murrina, especially during pyrexial rises.

In surra, murrina and nagana, several cc of blood and an equal quantity of 2 per cent sodium citrate in physiological salt solution may be centrifugalized at high speed for about fifteen minutes, the supernatant fluid carefully removed and the upper white stratum examined wet with the high dry lens. If trypanosomes are present, they are usually demonstrable. Animal inoculations may be required.

While the complement-fixation test is an excellent method of diagnosis, it is a group reaction, *i e*, an antigen prepared from one species of trypanosomes gives positive reactions with the sera of animals infected with any form of trypanosomiasis other than the non-pathogenic types and *T. cruzi*. Therefore the diagnosis, after a positive complement-fixation test, is based on the clinical symptoms and geographical prevalence of the disease. In the United States most antigens are prepared from *T. equiperdum*. (See section on antigen preparation.)

XV. Piroplasmosis.—Caused by: *Babesia equi* (*Piroplasma caballi*, *Zabesia bigemina*, *Piroplasma plasma canis*) (dog).—Until that two species of parasites *osis*—*Piroplasma caballi* and that these parasites are only *Babesia equi* is considered as the only known cause of piroplasmosis in horses.

Blood smears from the peripheral circulation should be prepared during a febrile period of the disease. When stained with Giemsa or Wright's the parasites appear within the erythrocytes as pear-shaped, ring-form, coccoid or rosette (four elements) structures. The pear-shaped forms are usually quite common and may occur singly, in pairs, and occasionally in groups of four.

In the case of canine piroplasmosis, large numbers (10 to 12) of the parasites are frequently found in a single red cell. They may be pear-shaped, and polyangular, and round extracellular forms may abound.

XVI. Filariasis of the Horse.—Caused by: *Filaria equina* (*F. papillosa*).—(Many kinds of filaria are found in the horse.) The same microscopic technic as that for the detection of trypanosomes may be employed or, instead of using citrate solution, the blood may be laked with distilled water, centrifugalized at high speed, the supernatant fluid discarded, and the residue examined. In making such examinations a number of specimens of blood should be taken at different times during a twenty-four hour period and repeated at intervals of four or five days before a negative diagnosis is made.

XVII. Filariasis of the Dog.—Caused by: *Dirofilaria immitis*. This parasite is called *Heartworm* because it is usually located in the right ventricle of the heart and the pulmonary artery of the dog causing shortness of breath and cyanosis. The larvæ appear in the circulating blood stream more abundantly at night. Diagnosis may be made by the method described for filariasis of the horse.

XVIII. Rabies.—Rabies is due to a neurotropic virus found in the saliva and in the nervous tissue of infected animals and is characterized by the formation of intracellular inclusions called Negri bodies. Laboratory diagnosis is based on examination of smears and histological sections of the

hippocampus major and cerebellum for Negri bodies and on animal inoculations of brain emulsions from the suspected animals. As Negri bodies are found in about 90 per cent of the cases an early diagnosis, in many instances, may be made by the simple touch preparations stained by Mann's or Sells's stain. If these are negative, animal inoculation should be performed.

If the brain material is fresh and uncontaminated it may be inoculated at once. If contaminated it should be placed in pure glycerol and held at refrigerator temperature for several days before subdural inoculation.

If touch preparations are negative, small portions of the hippocampus should be emulsified in several cc. of sterile physiological salt solution and about 0.03 cc. inoculated intracerebrally into white mice. This may be accomplished by forcing the hypodermic needle, with a drilling motion through the thin bone lying in the depression just posterior to the eye, using a $\frac{1}{4}$ -inch needle with a short, sharp bevel. The intralingual and intracerebral routes of injection into anesthetized rabbits may also be used.

As a rule the average rabies street virus will cause death in the mouse in nine to thirteen days and in the rabbit in about sixteen days; at times, longer. The animals usually develop the dumb form of rabies.

Negri bodies may be demonstrated in the brain tissue of the mice, rabbits and guinea pigs dying from rabies but they are usually smaller than those observed in the street cases and they are more easily distorted in making smears.

Histopathological examinations may be made by the paraffin method and stained with Mann's stain. Remove the paraffin with xylene, then pass the sections through absolute, 95, 75 and 50 per cent ethyl alcohol, and water, then stain. After staining, wash in water, then through 50, 75, 95 per cent, and absolute ethyl alcohol. Clear in equal parts of xylene and oil of cloves, and mount.

1. **Stains for Negri Bodies.**—Remove a small portion of the hippocampus major from the brain and place it on the cut surface of a small cork. Make "touch" preparations by touching a slide lightly to the surface of the tissue and pressing down lightly until the tissue spreads out. This will leave a thin film of tissue on the slide. Preparations made in this manner may be stained by either of the following methods:

Sells's Stain for Negri Bodies

	Cc.
Basic fuchsin, saturated solution in absolute methyl alcohol	2-4
Methylene blue, saturated solution in absolute methyl alcohol	15
Methyl alcohol, absolute, acetone-free	25

Mix the methylene blue solution and methyl alcohol, then add 2 cc. of the basic fuchsin solution. Make a trial stain by flooding "touch" preparations with the stain for five seconds; then wash in water, and dry in air. Blotting may remove part of the smear. Smears may be immersed in the stain in a Coplin jar.

A properly stained smear should be reddish-violet in the thinner areas, shading to purplish-blue in the thicker areas, when viewed by transmitted light. Microscopically, chromatin should stain blue, cytoplasm red and Negri bodies a very bright red. If a clear-cut differentiation is not obtained, add more fuchsin and make another trial. Usually 3 cc. of the fuchsin solution are sufficient.

The mixed stain improves on standing and keeps indefinitely if protected

against evaporation. If, because of evaporation, the stain produces too intense a red, the addition of absolute methyl alcohol will correct this fault.

Mann's Stain

	Cc.
Methyl blue (China blue) 1 per cent aqueous solution	3 5
Eosin, 1 per cent aqueous solution	3 5
Distilled water	10 0

The methyl blue and eosin solutions are kept in the refrigerator as separate stock at one time. A

The dye required is not methylene blue, but methyl or China blue.

"Touch" preparations are fixed in absolute methyl alcohol, washed in tap water and stained with the mixture for five to fifteen minutes, washed in water, dehydrated by passing rapidly through 50, 75, 95 per cent, and absolute ethyl alcohol, and cleared in xylene and cedar oil, equal parts. The Negri bodies stain brick red, the nerve cells blue, and red blood cells an orange color.

XIX. Equine Encephalomyelitis.—Equine encephalomyelitis is due to a pantropic virus occurring in the brain, lungs, spleen and liver of horses, mules, birds and man. It is only present in the blood stream during the early stages of the disease. Five distinct types are recognized, two of which occur in the United States, the Eastern and more virulent type, and the Western milder type. Laboratory diagnosis is based on neutralization and complement-fixation tests on blood serum from the suspected patient and on guinea pig protection tests with brain suspensions from dead animals. For the latter test, the brain should be received in a fresh well-preserved state. If contaminated with bacteria, it may be emulsified and passed through a Berkefeld "V" filter or a Seitz filter using an E.G. pad.

1. Guinea Pig Protection Test.—The brain material, in a dilution of 1 to 100, is injected intracerebrally into three groups of guinea pigs. One group should have been immunized against the Western type virus, the second group against the Eastern type virus, and the third group should be made up of normal animals. If equine encephalomyelitis of the North American type is present, all of the guinea pigs except those immunized against the specific type should die in three to six days.

2. Virus Neutralization Test.—The brains removed from mice moribund with the disease are ground with alundum, adding serum-hormone broth (pH 7.6) to make a 20 per cent suspension. The suspension is centrifugalized at 2500 r.p.m. for twenty minutes and the supernatant removed. The

to be tested. Mixture is brought about by shaking and the tubes placed in the incubator at 37° C. for one-half hour. For each serum-virus mixture selected four young albino Swiss mice are employed. Tuberculin syringes with 26-gauge needles are used to inoculate the mice intracerebrally with 0.03 cc. amounts of the serum-virus mixtures. Controls are run with known positive and negative sera. The mice are observed daily and deaths recorded for ten days.

XX. Virus Abortion in Mares.—(Dimock and Edwards.)—This disease usually occurs as an epizootic. The mares usually suffer no apparent

physical reaction except abortion. The aborted fetuses are usually bacteriologically negative. Characteristic gross lesions are small, multiple grayish-white areas of degeneration in the liver; hemorrhages in the heart, spleen and lungs and congestion of the colic lymph glands; and excessive amount of fluid in the thoracic cavity. A laboratory diagnosis can be made by the demonstration in cut sections stained with hematoxylin-eosin, of intranuclear acidophilic inclusion bodies that occur in the parenchymatous cells of the liver at the periphery of small areas of degeneration and in the respiratory epithelium lining the larger bronchioles of the lungs of aborted fetuses. Goodpasture has reported the passage of this virus, with its characteristic lesions, by the intraperitoneal inoculation of one-day-old Syrian hamsters.

XXI. Canine Distemper.—The demonstration of inclusion bodies in tissue sections and smears provides a rapid method for the diagnosis of this virus disease. Specimens should be obtained from an animal that is sacrificed in the advanced stages or recently dead from the disease. Cotton swabs are applied to the epithelium of the bladder and large bronchi and rubbed on clean glass slides. The slides are air dried or fixed in absolute methyl alcohol for ten minutes before staining. Tissue sections are prepared by the xylene-paraffin method. Preparations may be stained with hematoxylin and eosin. By this method the hematoxylin stains the cell walls, nuclei, and cytoplasm while the eosin stains the inclusion bodies red. The inclusion bodies vary in size, shape and number per cell and are usually located in the cytoplasm and at times in the nucleus. The presence of cytoplasmic artifacts may lead to error if they stain the same color and intensity as inclusion bodies.

TECHNIC OF THE COMPLEMENT-FIXATION TEST

Complement-fixation tests for the diagnosis of glanders, trypanosomiasis, equine encephalomyelitis and infectious abortion vary only in the specific antigens employed. The technic of the complement-fixation test for glanders, which is most widely used, is described in detail.

1. Preparation of Antigens.—**1. Glanders Antigen.**—The growth from forty-eight-hour cultures of *Malleomyces mallei* grown on large slants of 3 per cent glycerol agar of pH 6.8, is washed off with sterile distilled water, allowing 6 to 8 cc. per tube, and the suspension heated to 65° C. for one hour to insure destruction of the organisms. Phenol is added to 0.5 per cent concentration and the suspension shaken mechanically for one hour on each of seven days. Meanwhile the antigen must be kept in the refrigerator. At the expiration of this period the suspension is placed in sterile centrifuge tubes and centrifugalized for one hour at 2000 to 3000 revolutions per minute, and the clear supernatant fluid, which constitutes the stock antigen, is drawn off and stored in sterile amber bottles. The residue is discarded. For use, a 1 to 10, to 1 to 20 dilution of the stock antigen, depending upon its density, is prepared and titrated.

2. Equine Infectious Abortion Antigen.—A very satisfactory antigen is that prepared according to the method of Kelser. Inoculate 25 to 30 tubes of bouillon (pH 7.6) with a culture of *Salmonella abortus equina* and incubate them for forty-eight to seventy-two hours. Remove the cultures from the incubator and keep them in the dark, at room temperature for

a month, or even longer. These old cultures are then heated to from 60° to 65°C. for one-half hour and are then filtered through a Berkefeld "V" filter. The filtrate is put into a large-size enamel pan so that the fluid spreads out over the bottom of the pan to a very shallow depth. Then, by playing a current of air on the fluid, by means of an electric fan operating at high speed, the material is dried. The dry culture filtrate is then removed from the bottom of the pan by use of a safety razor blade held by a suitable handle. The collected material is further dried by placing it in a desiccator over sulfuric acid. After forty-eight hours the mass will be thoroughly dry and can then be pulverized in a dry mortar and put up in homeopathic vials. For titration, 150 mg. of the powdered antigen are dissolved in 10 cc. of physiological salt solution. The antigenic unit of such dilution is usually found to be 0.05 to 0.1 cc. Another, but less satisfactory type of antigen is prepared from *abortiroequina* in one-half hour. The antigen is then diluted with 1 per cent phenol. This

antigen should be titrated from time to time, one part of antigen and two parts of physiological saline solution being the usual dilution for titration.

3. *Trypanosomiasis* Antigen.—In the preparation of antigens for the *surra*, *murrina*, and *trypanosomiasis* are prepared from *T. equiperdum*. The trypanosomes are carried in rabbits or guinea pigs, which animals survive infection from one to two months. When it is desired to prepare an antigen, a drop of blood from the ear veins of such animals is examined microscopically to ascertain if trypanosomes are present in the circulation. At times, these animals apparently will be negative for several days, as the trypanosomes, especially *T. equiperdum*, make only periodic invasions of the peripheral circulation.

When such examinations prove positive, a small quantity of blood containing trypanosomes is diluted with salt solution and inoculated subcutaneously into one or two white rats. Usually in three to four days the blood of the inoculated rat will be found to contain a large number of trypanosomes. It can then be bled to death, catching the blood in a little physiological saline solution containing 1 per cent sodium citrate. This latter is then used to infect the desired number of rats for antigen production.

As a rule, the average inoculation will cause death in the rat in about three to four days, therefore it is necessary to examine the blood at about twelve-hour intervals after the second day. This may be done by snipping off a small portion of the tail. When the blood is teeming with trypanosomes, the rats should be bled to death by severing the blood vessels of the neck with a sharp scalpel or razor. The blood is collected in a quantity of physiological salt solution containing 1 per cent sodium citrate (about 100 cc. for 12 rats). When all the blood has been collected, the mixture is filtered through cheesecloth to remove small clots, hair, etc.; poured into tubes, and centrifugalized for about thirty minutes at 2500 revolutions per minute. This sediments the corpuscles and trypanosomes. The supernatant fluid is drawn off and discarded. To the mass of corpuscles intermixed with and overlaid by trypanosomes, is added sufficient distilled water (200 cc. for 12 rats) to produce complete hemolysis of the erythrocytes, a matter of about twenty minutes, which procedure is facilitated by

agitation of the mixture in a flask. This is centrifugalized for about one-half hour, upon the completion of which there is found at the bottom of the tubes a mass of trypanosomes with an admixture of stroma of the hemolyzed red cells.

After discarding the supernatant fluid (hemoglobin-stained water) physiological salt solution is added, and the material vigorously shaken until the mass of trypanosomes is disintegrated and evenly distributed throughout the solution. Centrifugation is again resorted to with similar results, the washed mass of trypanosomes being packed at the bottom of the tubes. The salt solution is drawn off and an amount of preserving fluid (equal parts of physiological salt solution and glycerol) equal to about twice the amount of trypanosomes added; the mixture is then agitated until a uniform suspension is produced. The antigen is stored at a low temperature, preferably in the freezing chamber of an electric refrigerator, until used. It should be titrated from time to time, 1 part of antigen and 10 parts of physiological salt solution being the usual dilution for titration. It will keep approximately two months.

4. **Equine Encephalomyelitis Antigen.**—The method of Casals and Palacios is followed: Infected mouse brains are ground in a mortar, diluted with physiological salt solution to ten times their volume by weight, and held in the icebox for twenty hours. After this time the emulsion is centrifugalized for thirty minutes at 2500 r.p.m. The supernatant is removed and frozen and thawed five times in a dry ice-alcohol mixture. It is then centrifugalized in an angle centrifuge for one hour at 3500 r.p.m. The supernatant is removed and preserved with merthiolate in a dilution of 1 to 10,000. This is the antigen. It is used undiluted in 0.25 cc. amounts and retains its antigenic properties for approximately two months.

II. **Preparation of the Hemolytic System.**—Hemolytic amboceptor, complement and sheep cells are prepared as described in Chapter XII, Sero-diagnosis of Syphilis.

Complement must be titrated on the same day used. Antigen and amboceptor titrations are made following their preparation and remain fairly stable.

1 guinea pig serum diluted to
5 pt washed sheep cells, 3 per cent
susp. 30, set up a series of tubes as
indicated in the following table.

TABLE 93—TITRATION SYSTEM FOR AMBOCEPTOR

Tube No.	Amboceptor 1-1000, cc.	Complement 5%, cc.	Sheep blood cells 3%, cc.	Salt solution 0.85%, cc.
1	1 0	1 0	1 0	2 0
2	0 8	1 0	1 0	2 0
3	0 7	1 0	1 0	2 0
4	0 6	1 0	1 0	2 0
5	0 55	1 0	1 0	2 0
6	0 5	1 0	1 0	2 0
7	0 46	1 0	1 0	2 0
8	0 43	1 0	1 0	2 0
9	0 33	1 0	1 0	2 0
10	0 1	1 0	1 0	2 0
11	1 0	0	1 0	2 0
12	0	1 0	1 0	2 0

The last two tubes are controls on amboceptor and complement, and neither should show hemolysis. Shake the tubes and place the rack con-

taining them in the 37.5° C. incubator or water bath for one hour at the end of which time note the smallest amount of amboceptor necessary to cause complete hemolysis of the red blood cells. This amount is known as the amboceptor unit. For the test, two units are employed, so diluted that this amount is contained in 1 cc. The amboceptor is best preserved by the addition of an equal amount of neutral glycerol to the serum. This is added previous to determining the hemolytic value of the amboceptor.

2. Titration of Complement.—A rack containing 15 tubes is set up and 2 cc. of physiological salt solution are placed in each tube. Then 0.5 cc. of the guinea pig serum (complement) are added to a tube containing 12 cc. of physiological salt solution, making a 4 per cent solution of complement. Of this mixture the following amounts are placed in the 15 test tubes: 0.3 cc., 0.35 cc., 0.4 cc., 0.45 cc., 0.5 cc., 0.55 cc., 0.6 cc., 0.65 cc., 0.7 cc., 0.75 cc., 0.8 cc., 0.85 cc., 0.9 cc., 0.95 cc., and 1 cc. One cc. of hemolytic amboceptor which has been so diluted with physiological salt solution that it represents 2 units is added to each of the 15 tubes and this is followed by the addition of 1 cc. of a 3 per cent suspension of washed red cells of a sheep to each of the tubes. The tubes are then incubated at 37.5° C. for one-half hour. The smallest amount of complement which causes complete hemolysis of the red cells is noted and one and a half times this amount is used for the test. The complement is so diluted with physiological salt solution that the 1.5 units are contained in 1 cc.

Example.—If complete hemolysis occurs in the seventh tube containing 0.6 cc. of the 4 per cent complement, the percentage would be determined in the : : : : : cc 0.6 equals 2.1 (the unit) plus on : : : : : .

In the : : : : : complement would be prepared and 1 cc. employed in the test.

3. Titration of Antigen.—For this purpose a double-row rack containing 24 test tubes, is set up, with each test tube containing 2 cc. of physiological salt solution. After adding positive and negative horse sera, the tubes are placed in a water bath at 57.5° C. for one-half hour to inactivate the sera. Sera used for controls may be preserved with 0.5 per cent phenol. The tubes are cooled, antigen and complement added as indicated in Table 94 and then incubated at 37.5° C. for one hour.

TABLE 94.—TITRATION OF ANTIGEN

Tube No.:	1	2	3	4	5	6	7	8	9	10	11	12
<i>Back Row of Tubes</i>												
Negative serum, cc.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.0
Antigen, cc.	0.1	0.2	0.3	0.4	0.5	0.6	0.8	1.0	1.2	0.0	0.5	0.0
1½ units complement, cc.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0
<i>Front Row of Tubes</i>												
Positive serum, cc.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.0
Antigen, cc.	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	0.6	0.0	0.0	0.0
1½ units complement, cc.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

The hemolytic system is then added, 1 cc. of 3 per cent suspension of sheep red blood cells to each tube, and two units of amboceptor contained in 1 cc. to each tube except the last tube of the front row. This is a control tube on the complement. Tube 12 in the back row containing no complement is a control on the amboceptor only. Tube 11 in the front row is a control on the hemolytic system only, tube 11 in the back row is a control on the antigen, and tubes 10 in each row are controls on positive and

negative sera respectively. Of the six control tubes, only the last tube in each row should show no hemolysis.

At the expiration of the second incubation period the titration is read. The smallest amount of antigen which has caused complete fixation of complement in the presence of positive serum and which has not interfered with hemolysis in the presence of negative serum is termed the "unit of antigen." In the application of the test twice the antigenic unit is employed provided twice that amount (4 units) does not inhibit hemolysis in the presence of negative serum. The 2 units of antigen should be so diluted with physiological salt solution that they are included in 0.5 cc. In the case of some antigens of wide range, greater amounts of antigen may be employed.

III. Technic of the Test.—1. **Apparatus.**—For the test, double-row metal racks capable of holding 24 tubes are employed. Six tubes are placed in the back row of the rack leaving an empty space between each tube. In the front row a tube is placed in each of the 12 holes. One rack is used for every 6 specimens of serum; the 6 tubes in the back row serve as serum controls and each contains 0.2 cc. of the particular serum, while in the front row 2 tubes are used for each specimen, one containing 0.2 cc. and the other 0.1 cc. of the serum under test.

2. **Procedure.**—Place 2 cc. of physiological salt solution in each of the tubes, then add the serum. Sera from horses, bovines and guinea pigs are inactivated in the water bath at 57.5° C. for thirty minutes, while sera from mules and rabbits are inactivated at 62° C. for forty minutes.

After inactivation allow the tubes to cool, then add the proper amount of antigen to each tube in the front row of the rack, omitting it in the tubes in the back row (serum controls).

To each tube add 1 cc. of complement which is so diluted with physiological salt solution that it contains 1.5 units.

Incubate at 37.5° C. for one hour; then add the hemolytic amboceptor, which has been so diluted with physiological salt solution that the two units are contained in 1 cc. and finally 1 cc. of a 3 per cent suspension of washed blood cells from the same suspension employed in the complement titration.

Incubate at 37.5° C. for one hour, then read the test.

3. **Reading and Interpreting the Results of the Test.**—In recording the results of the test the following terms are used: Where there is complete fixation of complement, results are indicated by 4 plus sign (++++). Positive cases give complete fixation in 0.2 cc. of serum and in the large percentage of cases in 0.1 cc. In incomplete fixation, the degree is noted as follows: 75 per cent fixation: 3 plus sign (+++); 50 per cent fixation: 2 plus sign (++) ; 25 per cent fixation: 1 plus sign (+). Where there is 50 per cent or more fixation the case is considered suspicious. Less fixation than this is considered slightly suspicious or negative depending on how well the control tubes have cleared up, and whether disease is known to be present in a locality. Complete hemolysis is negative.

Should hemolysis fail to occur in both antigen and serum control tubes, the serum is termed anti-complementary and fresh specimens will be required.

The following controls are carried out with the test: Antigen control, positive serum control, negative serum control, and the complete hemolytic system.

PART XI

Statistical Methods

By PAUL R. HAWLEY

THIS section is designed for the laboratory worker, interested in fields other than mathematics, who occasionally requires tools of this nature in the analysis of his experimental data. Insofar as practicable, the formulas have been expressed in arithmetic, rather than algebraic, form so that a working knowledge of ordinary arithmetic is all that is required to follow these procedures. Even the usual symbolism of biostatistics has been altered where my experience has shown it to be confusing to the average student.

These pages pretend to be nothing more than a guide to the practical application of a few simple statistical procedures. The reader who is interested in statistical theory, is referred to the works of Pearl, Elderton, Yule, King, *et al.*, which have been freely consulted in the preparation of the following chapters.

I am deeply indebted to my old friend and preceptor, Dr. Lowell J. Reed, for advice upon several points that have arisen in compiling this section.

CHAPTER XLIV

RATES AND RATIOS

RATES

MOST rates are probabilities in retrospect. The basic general formula for all rates is

$$\text{Rate} = \frac{\text{Number experiencing a specific event}}{\text{Number that might have experienced the specific event}}$$

If the numerator of the above be designated by the symbol f , and the denominator by n , we have

$$R = \frac{f}{n}$$

It is customary, in vital statistics, to express rates in terms of occurrences per 1,000, per 10,000, per 100,000, or in general, per K of those exposed to the occurrence of the event. The general formula then becomes

$$R = \frac{K \times f}{n}$$

which is to say that there are R occurrences of f per K of n .

In vital statistics n is the entire population or some specified part thereof. When n is the entire population, and f the number of events in the entire population, the rates so derived are known as

Crude Rates.—

$$\text{Crude death-rate} = \frac{K \times \text{number of deaths}}{\text{population}}$$

$$\text{Crude morbidity-rate} = \frac{K \times \text{number of cases of disease}}{\text{population}}$$

$$\text{Crude birth-rate} = \frac{K \times \text{number of births}}{\text{population}}$$

Since vital rates measure vital forces—force of mortality, force of morbidity, force of natality, etc.—the measurement is necessarily “crude” unless n be a homogeneous group of which all the individuals are more or less equally exposed to the risk of experiencing the specified event. The force of mortality from puerperal eclampsia, for example, is very poorly measured by a rate wherein n is the entire population. The only individuals at all exposed to the risk are the gravid females. To a lesser degree, the death-rate from all causes. All risk of dying within a specified time. Age, sex, race, occupation, etc., all influence the risk of dying.

The significance of the measurement of vital forces is increased by means of

Specific Rates.—In specific rates, both f and n are defined and limited as to specified attributes, *e. g.*, age, sex, color, race, occupation, marital status, etc., or any combination thereof; and R is designated an age-specific rate, an age-sex-specific rate, a sex-color-specific rate, etc., as the case may be. These rates are computed by the general formula, except that f and n include only specified individuals. For example, the specific death-rate for males, aged forty years, is

$$\text{Death-rate}_{m-40} = \frac{K \times \text{deaths in males, aged forty}}{\text{number of males, aged forty years, in population}}$$

Since certain attributes of populations exert great influence upon the force of mortality, a comparison of the force of mortality in two or more groups—cities, for example—through the medium of crude death-rates will obviously be misleading when the several populations are unequally distributed as to age, sex, color, etc. Take, as examples, Baltimore, Md., with a slow-growing population that includes a high proportion of colored; Detroit, Mich., a rapidly growing industrial city with a high proportion of young adult males; and Los Angeles, Calif., another city of rapid growth but whose population includes an unusual proportion of retired, elderly people attracted by the equable climate. Elderly people are exposed to the risk of dying to a greater degree than young adults, even in an optimum climate, and negroes experience, at most ages, higher mortality than whites. If the force of mortality in Los Angeles were exactly that in Detroit, the crude death-rate of the former would be appreciably higher because of its larger proportion of aged; and the crude death-rate of Baltimore is increased by its large colored population.

The significance of comparisons of the force of mortality in two or more populations is increased in proportion to the specificity of the rates used; but it is apparent from Table 96 that such comparisons would be difficult of expression when a number of specific rates are necessary. However, this difficulty is overcome by means of:

Corrected Death-rates.—A corrected death-rate is a *hypothetical* (crude) rate that would obtain if the same force of mortality were exerted in a population of selected distribution with regard to specified attributes. Death-rates may be corrected for age, sex, race, etc., just as specific rates may be computed for any attribute or combination of attributes of population. Corrected death-rates are derived by applying the specific death-rates of the populations in question to a standard population; from the summation of the hypothetical deaths thus obtained, a crude rate is computed for the standard population; and this crude rate for the standard population is the corrected rate for the population in question.

TABLE 95 — POPULATION AND DEATHS, BY AGES AND COLOR, FOUR AMERICAN CITIES, 1920

Ages	Population		Deaths		Population		Deaths	
	White	Colored	White	Colored	White	Colored	White	Colored
Baltimore								
Detroit								
Under 1	12,884	1,608	1,469	472	21,673	595	2,734	151
1-4	48,097	6,407	407	185	87,930	1,919	1,181	77
5-9	56,423	8,069	162	39	86,044	2,201	438	18
10-19	107,776	15,934	267	118	134,194	4,664	531	50
20-39	219,641	47,649	1,341	655	416,115	24,607	2,951	481
40-59	131,472	23,433	1,921	700	162,597	6,662	2,197	215
60-79	45,601	4,821	2,532	358	39,833	707	2,100	60
80 and over	3,126	354	572	49	2,630	47	527	8
Sub-total	625,020	108,295	8,781	2,574	951,316	41,602	12,659	1,040
Total	733,315		11,355		992,918		13,699	
Los Angeles								
New Orleans								
Under 1	6,784	765	741	89	4,831	1,506	435	350
1-4	28,126	2,453	310	40	19,733	6,224	167	105
5-9	37,632	1,999	164	11	26,091	8,843	81	46
10-19	72,051	3,130	281	21	53,461	18,325	172	174
20-39	201,755	13,307	1,332	157	103,806	41,764	792	826
40-59	143,600	5,046	1,539	141	58,330	19,628	1,077	743
60-79	52,413	1,022	2,421	67	17,878	4,054	1,192	363
80 and over	4,003	73	647	13	1,370	510	264	50
Sub-total	546,584	28,695	7,735	539	285,500	100,944	4,180	2,667
Total	575,279		8,274		386,444		6,867	

Table 95 gives the population and the deaths, by age and color, for Baltimore, Detroit, Los Angeles and New Orleans for the year 1920. The crude death-rates per thousand are:

$$\text{For Baltimore: Crude death-rate} = \frac{1,000 \times 11,355}{733,315} = 15.48$$

$$\text{For Detroit: Crude death-rate} = \frac{1,000 \times 13,699}{992,918} = 13.80$$

$$\text{For Los Angeles: Crude death-rate} = \frac{1,000 \times 8,274}{575,279} = 14.38$$

$$\text{For New Orleans: Crude death-rate} = \frac{1,000 \times 6,867}{386,444} = 17.77$$

The age and color specific death-rates are given in Table 96.

TABLE 96.—AGE AND COLOR SPECIFIC DEATH-RATES FROM DATA IN TABLE 95

Ages	Baltimore		Detroit		Los Angeles		New Orleans	
	White	Colored	White	Colored	White	Colored	White	Colored
Under 1	115 57	293 53	126 15	253 78	109 23	116 34	90 04	219 30
1-4	10 33	28 87	13 43	40 13	11 02	16 31	8 46	16 87
5-9	2.87	4 83	5 09	8 18	4 36	5 50	3.10	5 20
10-19	2 48	7 27	3 96	10 72	3 90	6 71	3 22	9 50
20-39	6 11	13 75	7 09	18 58	6 60	11 50	7.63	19 82
40-59	14 61	29 87	13 49	32 27	12 79	23 71	18 46	37 85
60-79	55 53	74 26	52 72	84 87	46 19	65 56	66.67	89 54
80-over	182 98	138 42	200 38	170 21	161 63	178 08	192 70	156 86
all ages*	14 05	23 77	13 31	25 00	14 15	18 78	14 64	26 62

* Specific for color only

The standard population may be any one of the four populations of Table 95, the population of the U. S. Registration Area by age and color, or any other actual or assumed population distribution. The important thing is that the specific rates of all the populations to be compared be applied to the same standard population. However, labor is saved by selecting one of the populations in question as the standard.

Selecting Baltimore as the standard, the age and color specific rates for the three other cities are applied to the Baltimore population and the hypothetical numbers of deaths that would have occurred, had these rates obtained in Baltimore, are set down in Table 97. From the totals of these hypothetical deaths are derived rates with the total population of Baltimore as the denominator of the fraction, thus—

For Detroit

$$\text{Corrected death-rate}_{ac} = \frac{1,000 \times 12,358}{733,315} = 16.85$$

For Los Angeles

$$\text{Corrected death-rate}_{ao} = \frac{1,000 \times 10,285}{733,315} = 14.03$$

For New Orleans:

$$\text{Corrected death-rate}_{nc} = \frac{1,000 \times 12,808}{733,315} = 17.47$$

and, of course, for Baltimore, the corrected rate is the same as the crude rate since the population of Baltimore was used as the standard. The relative position of Detroit is changed by correction of the death-rates for age and color. It is apparent that the corrected death-rates will vary according to the standard population used and that they are significant only as comparative figures in the designated comparison.

Standardized Death-rates.—A standardized death-rate is a hypothetical (crude) rate derived by applying the specific death-rates of the U. S. Registration Area (or any other arbitrarily selected population) to a given population and computing the general rate from the summation of the hypothetical deaths. The procedure is similar to the computation of corrected death-rates except that the specific rates are those of the standard population and the population is the actual population for which the standardized rate is to be derived.

TABLE 97.—NUMBER OF DEATHS THAT WOULD HAVE OCCURRED IN BALTIMORE HAD THE AGE AND COLOR SPECIFIC RATES OF THREE OTHER CITIES OBTAINED

Ages	Age-color specific rates of					
	Detroit		Los Angeles		New Orleans	
	White	Colored	White	Colored	White	Colored
Under 1	1,625	408	1,407	187	1,160	353
1-4	646	257	530	104	407	108
5-9	287	66	246	44	175	42
10-19	427	171	420	107	347	152
20-39	1,557	885	1,450	562	1,676	944
40-59	1,774	756	1,682	556	2,427	887
60-79	2,404	409	2,106	316	3,040	432
80 and over	626	60	505	63	602	56
Sub-total	9,346	3,012	8,346	1,939	9,834	2,974
Total	12,358		10,285		12,808	

Probable Error of Rates.—The probable error of f (the expected number of occurrences) is

$$PE_f = 0.67449 \times \sqrt{n \times p \times q}$$

where n is the number of trials, p the probability that the event will, and q the probability that the event will *not*, happen as specified. But, in this case,

$$p + q = 1.0$$

In rates per K ,

$$\frac{P}{Q} = \frac{K}{K - P}$$

and the probable error of the rate becomes

$$PE_R = 0.67449 \times \sqrt{\frac{n \times P \times Q}{n}}$$

But,

$$\frac{\sqrt{n}}{n} = \frac{1}{\sqrt{n}}$$

Hence,

$$PE_R = 0.67449 \times \sqrt{\frac{P \times Q}{n}}$$

In the specific example of the crude death-rate for Baltimore for the year 1920, we have

$$\begin{aligned} PE_R &= 0.67449 \times \sqrt{\frac{15.48 \times 984.52}{733,315}} \\ PE_R &= 0.67449 \times \sqrt{0.0207829417} \\ &= 0.67449 \times 0.14416 \\ &= 0.097 \end{aligned}$$

and the crude death-rate is expressed thus:

$$CR \text{ Baltimore, 1920} = 15.48 \pm 0.097$$

Where the rate is small, a more accurate approximation of the probable error will be had from the following formula:

$$PER' = 0.67449 \times K \times \frac{\sqrt{f}}{n}$$

Applying this formula to the crude death-rate of Baltimore, we have

$$\begin{aligned} PER' &= 0.67449 \times 1,000 \times \frac{\sqrt{11,355}}{733,315} \\ &= 674.49 \times \frac{106.56}{733,315} \\ &= 0.098 \end{aligned}$$

In this particular instance, both formulas give approximately the same result. As R decreases in size, the advantages of the latter formula increase.

Infant Mortality-rate.—The infant mortality-rate is derived by the general formula wherein f is the number of deaths under one year of age and n is the number of living births during the same period.

Case Fatality-rate.—The case fatality-rate is the proportion of deaths from a disease to the number of cases of the disease. Thus, f = the number of deaths and n = the number of cases. This rate usually being expressed as a *per cent*, $K = 100$, unless otherwise specified.

If, of 69 cases of lobar pneumonia, 23 died, the

$$\begin{aligned} \text{Case Fatality-rate} &= \frac{100 \times 23}{69} \\ &= 33.33 \text{ (per cent)} \end{aligned}$$

The probable error would be

$$\begin{aligned} PE_{cfr} &= 0.67449 \times \sqrt{\frac{33.33 \times 66.67}{69}} \\ &= 3.83 \end{aligned}$$

In *mortality-rates*, the exposed to risk group includes all those exposed to the risk of contracting the disease; whereas, in *case fatality-rates*, the exposed to risk group includes only those who have already contracted the disease.

RATES USED IN THE MILITARY SERVICES

Admission Rates.—The force of morbidity is expressed in the military services in terms of admissions (to sick report) per 1,000 per annum. Regardless of the duration of the experience, the rate assumes it to have been one year (per annum). The rate states, in plain language, that, had the admissions from the specified cause or causes continued at the same level for one year, there would have been Ar admissions in each 1,000 (K) of mean, or average, strength of command.

The general formula for all rates, set down at the beginning of this chapter, is modified only by the introduction of the factor of time. The formula for the admission-rate becomes

$$\begin{aligned} Ar &= \frac{K \times f \times T}{n \times t} \\ Ar &= \frac{1,000 \times f \times T}{n \times t} \end{aligned}$$

where T = the theoretical duration of the experience (= one year)
and t = the actual duration of the experience

It is, of course, essential that T and t be expressed in the *same* units of time—months, weeks or days. T will always be either 12, 52 or 365, depending upon whether t is expressed as months, weeks or days, respectively.

For example, in a command with a mean strength of 3,467, there were 254 admissions to sick report from all causes during the month of February. The admission rate for the month of February is

$$\begin{aligned} Ar &= \frac{1,000 \times 254 \times 12}{3,467 \times 1} \\ &= \frac{3,048,000}{3,467} \\ &= 879.1 \end{aligned}$$

which is to say that the admission-rate from all causes for the month of February was 879.1 per 1,000 per annum.

Another example: From February 1 to February 14, inclusive, in the same command there were 131 admissions. The admission rate per 1,000 per annum for the first two weeks of February would be

$$\begin{aligned} Ar &= \frac{1,000 \times 131 \times 52}{3,467 \times 2} \\ &= \frac{6,812,000}{6,934} \\ &= 982.4 \end{aligned}$$

Since the admission-rate may, and frequently does, exceed 1,000 per 1,000 per annum, it is apparent that it is not a probability of occurrence but is rather an average of occurrences. The probable error of the admission-rate is, therefore, derived by the same process as shown in the second example under Probable Error of Rates, above. It is

$$PE_{Ar} = 0.67449 \times K \times T \times \frac{\sqrt{I}}{n \times t}$$

Applying this to the second example of an admission-rate, we have

$$\begin{aligned} PE_{Ar} &= 0.67449 \times 1,000 \times 52 \times \frac{\sqrt{131}}{3,467 \times 2} \\ &= 674.49 \times 52 \times \frac{11.445522}{6,934} \\ &= 57.59 \end{aligned}$$

Non-effective Rates.—Loss of time from disease and injury is measured, in the military services, by non-effective rates. The same general principles apply: The non-effective rate is the ratio of days lost from disease or injury to the total number of days that could have been lost had every man been incapacitated every day. It is expressed in terms of 1,000 mean strength (*per K*), and the formula is

$$Ner = \frac{1,000 \times \text{days lost}}{n \times \text{days of experience}}$$

particular distribution. This is to say that great difficulties are encountered when such irregular intervals as 0 to 4, 5 to 8, 9 to 17, 18 to 24, 25 to 33, etc., are used. Occasionally data are available only in such form and the statistician, like any other workman, must make the best of the material

The *mid-point* of a class interval is that point on the abscissa that lies equidistant from the two limits of the interval. The mid-point is occasionally referred to as the *class mark*. The beginner sometimes forgets that intervals are designated by inclusive numbers, and may place the mid-points in wrong positions. For example, the age interval of five to nine years is one of five years, and the mid-point is at 7.5; and the interval of 0 to 2 is one of three years, with the mid-point at 1.5 years.

ANALYSIS OF FREQUENCY DISTRIBUTIONS

There are several characteristics of every distribution that serve to identify it just as height, weight, girth and posture are characteristics that identify a man and distinguish him from other men. There are many men of equal height, of equal weight, or having the same chest measurements, etc.; but there are very few men all of whose measurements are identical with the measurements of another man. This is the basis of the Bertillon system of identification.

And so it is with frequency distributions. Two distributions may have certain characteristics in common; but rarely do two distributions have all their distinguishing characteristics each like the other unless they be both representative samples of the same variable. In this manner do we contrast and compare variable phenomena.

For expositions of the theories upon which the steps in the analysis of frequency distributions are based, the student may consult any standard text upon statistical methods. The steps, themselves, are given below.

Moments About the Arbitrary Axis.—The first step in the location of the mean is the selection of an arbitrary axis as a point of departure for further calculations. This point may be located, at will, any place within or without the range of variation. It is a point of trial, arbitrarily selected, of the rotational characteristics of the distribution.

In the example given in Table 98, the point selected is at 163.5. This is just outside the range of the data. Examples of the computations when the point selected is within the range of the data are given in Chapter XLVI.

The distance of the arbitrary axis from the mid-points of each of the class intervals is then determined by algebraic subtraction. It is obvious that, if the arbitrary axis be placed at any point on the abscissa of greater numerical value than one or more of the mid-points, some of the values of X will be negative. This does not alter the principles in the least and the computations are the same, having regard for the sign of X .

It usually simplifies computations to substitute smaller numbers, with the same relationships, for the values of X . In the example given, the true value of $X_{188.5}$ is, of course, $188.5 - 163.5 = 25$. But the powers of 25 run

into large figures, to say nothing of the powers of $463.5 - 163.5 = 300$. These so-called working units may be converted into actual units after the computations are made.

TABLE 98.—FREQUENCY DISTRIBUTION OF BODY-WEIGHTS OF HEALTHY INDIAN PIGEONS*

Class intervals Weight in grams	Mid- points X	Work units z	Variate fre- quencies Z	Variate frequencies multiplied by powers of x			
				Zx	Zx^2	Zx^3	Zx^4
151-175	163.5	0	0	0	0	0	0
176-200	188.5	1	3	3	3	3	3
201-225	213.5	2	25	50	100	200	400
226-250	238.5	3	45	135	405	1,215	3,645
251-275	263.5	4	60	240	960	3,840	15,360
276-300	288.5	5	84	420	2,100	10,500	52,500
301-325	313.5	6	76	456	2,736	16,416	98,496
326-350	338.5	7	42	294	2,058	14,406	100,842
351-375	363.5	8	41	328	2,624	20,992	167,936
376-400	388.5	9	16	144	1,296	11,664	104,976
401-425	413.5	10	2	20	200	2,000	20,000
426-450	438.5	11	3	33	363	3,993	43,923
451-475	463.5	12	1	12	144	1,728	20,736
Σ (the sum of)			398	2,135	12,989	86,957	628,817

* Data from McCarrison and Sundarajan *Beri-beri Columbarum*, Calcutta, Thacker, Spink & Co., 1928.

$$\begin{aligned}
 \text{First moment: } \mu_1 &= \frac{\Sigma Zx}{\Sigma Z} = \frac{2,135}{398} = 5.36432 \\
 \text{Second moment: } \mu_2 &= \frac{\Sigma Zx^2}{\Sigma Z} = \frac{12,989}{398} = 32.6357 \\
 \text{Third moment: } \mu_3 &= \frac{\Sigma Zx^3}{\Sigma Z} = \frac{86,957}{398} = 218.485 \\
 \text{Fourth moment: } \mu_4 &= \frac{\Sigma Zx^4}{\Sigma Z} = \frac{628,817}{398} = 1,579.94
 \end{aligned}$$

Moments About the Mean:*

$$\begin{aligned}
 \text{First moment } \phi_1 &= 0 \\
 \text{Second moment: } \phi_2 &= \mu_2 - \mu_1^2 \\
 &= 32.6357 - 28.77594 \\
 &= 3.85977 \\
 \text{Third moment: } \phi_3 &= \mu_3 - 3\mu_1\mu_2 + 2\mu_1^3 \\
 &= 218.485 - 525.20502 + 308.72658 \\
 &= 2.00656 \\
 \text{Fourth moment: } \phi_4 &= \mu_4 - 4\mu_1\mu_2 + 6\mu_1^2\mu_2 - 3\mu_1^4 \\
 &= 1,579.94 - 4,688.0938 + 5,634.7357 - 2,484.1622 \\
 &= 424.197
 \end{aligned}$$

Corrected Moments About the Mean.—The grouping of variate frequencies into the several class intervals introduces error in the moments about the mean through the assumption that all the frequencies are located at the respective mid-points. This error is corrected by Sheppard's corrections, which are applicable when there is high contact at both ends of the dis-

* The usual symbolism has been abandoned so that, for the casual student, x may always mean 3 1416.

tribution—i. e., when the frequencies tend to tail off regularly, on a curve asymptotic to zero.

$$\begin{aligned}
 \text{First moment: } \mu_1 &= \phi_1 = 0 \\
 \text{Second moment: } \mu_2 &= \phi_2 - \frac{1}{2} \\
 &= 3.85977 - 0.08333 \\
 &= 3.77644 \\
 \text{Third moment: } \mu_3 &= \phi_3 = 2.00656 \\
 \text{Fourth moment: } \mu_4 &= \phi_4 - \frac{1}{2}\phi_2 + \frac{3}{8}\sigma^2 \\
 &= 42.4197 - 1.9299 + 0.0292 \\
 &= 40.5190
 \end{aligned}$$

The Betas.—There are two functions of the moments about the mean that simplify computations in the measurement of skewness, to follow.

$$\begin{aligned}
 \beta_1 &= \frac{\mu_3^2}{\mu_2^3} \\
 &= \frac{4.02628}{53.8577} \\
 &= 0.074758 \\
 \beta_2 &= \frac{\mu_4}{\mu_2^2} \\
 &= \frac{40.519}{14.2615} \\
 &= 2.84115
 \end{aligned}$$

The Standard Deviation.—The standard deviation is the measure of scatter or dispersion of the variate frequencies

$$\begin{aligned}
 \sigma &= \sqrt{\mu_2} \\
 &= \sqrt{3.77644} \\
 &= 1.94331 \text{ (in working units)}
 \end{aligned}$$

But each working unit represents 25 grams of weight, so that, in terms of variation in weight,

$$\begin{aligned}
 \sigma &= 25 \times 1.94331 \\
 &= 48.58275 \text{ (in grams of weight)}
 \end{aligned}$$

Probable Error of the Standard Deviation.

$$\begin{aligned}
 PE_\sigma &= \frac{0.67449\sigma}{\sqrt{2N}} \\
 \text{Where } N &= \text{number of variate frequencies:} \\
 PE_\sigma &= \frac{0.67449 \times 48.58275}{\sqrt{2 \times 398}} \\
 &= \frac{32.76858}{28.21347} \\
 &= 1.16145
 \end{aligned}$$

The Mean.—Since, by its very nature, the first moment about the mean must equal zero, the arbitrary axis is removed from the mean by the distance represented by the first moment about the arbitrary axis (ν_1).

$$\begin{aligned}
 \nu_1 &= 5.36432 \text{ (in working units)} \\
 &= 5.36432 \times 25 \\
 &= 134.108 \text{ (in grams of weight)} \\
 \text{Hence, The Mean} &= 163.5 + 134.108 \\
 &= 297.608
 \end{aligned}$$

Probable Error of the Mean.

$$\begin{aligned}
 PE_{\text{mean}} &= \frac{0.67449\sigma}{\sqrt{N}} \\
 &= \frac{0.67449 \times 48.58275}{\sqrt{398}} \\
 &= \frac{32.76858}{19.94994} \\
 &= 1.643
 \end{aligned}$$

which is to say that the mean weight of the 398 pigeons was 297.6 ± 1.64 grams.

The Median.—The *median* is that point on the abscissa on either side of which lie one-half the total frequencies.

There are 398 frequencies in this distribution. On either side of the median must lie exactly 199 frequencies. Starting the count at the lower end of the range, there are in the several class intervals

$$3 + 25 + 45 + 60 = 133$$

frequencies in class intervals 1 to 4, inclusive. There are 84 frequencies in interval number 5, so that frequency number 199 lies somewhere within the fifth class interval (276 to 300). But, we only want to go into the fifth class interval $199 - 133$, or 66 frequencies to the median. The theory of frequency distributions assumes frequencies to be equally distributed

25 gm. of weight. So,

$$\begin{aligned}
 \text{Median} &= 276 + (66/84 \times 25) \\
 &= 276 + 19.64 \\
 &= 295.64 \text{ (grams of weight)}
 \end{aligned}$$

Probable Error of Median.

$$\begin{aligned}
 PE_{\text{median}} &= 1.25332 \times PE_{\text{mean}} \\
 &= 1.25332 \times 1.643 \\
 &= 2.059
 \end{aligned}$$

and the median is 295.6 ± 2.06 grams.

The Mode.—The *mode* is the point (on the abscissa) of maximum frequency of occurrence, *i. e.*, the fashion, the custom. In strictly normal distributions, the mode must coincide with the mean and the median. When the mode lies to one side or the other of the mean by a significant distance, the distribution is said to be *skew*.

Skewness.—Skewness is asymmetry of distribution, *i. e.*, where the slope of the plot of the distribution is steeper on one end of the range than on the other

$$\begin{aligned}
 \text{Let } d &= \text{Mean} - \text{Mode} \\
 \text{and } d &= x \times \sigma
 \end{aligned}$$

$$\text{Whence, } x = \frac{d}{\sigma}$$

x (chi) is the mathematical measure of skewness and, in terms of moments,

$$x = \frac{\sqrt{\beta_1} \times (\beta_2 + 3)}{2 \times (\beta_3 - 3\beta_1 - 9)}$$

$$\begin{aligned}
 &= \frac{\sqrt{0.074758 \times 5.84115}}{2 \times (14.20575 - 0.418548 - 9)} \\
 &= \frac{0.273419 \times 5.84115}{2 \times 4.757202} \\
 &= \frac{1.597081}{9.514404} \\
 &= 0.167859
 \end{aligned}$$

$$\begin{aligned}
 \text{Now, } d &= x \times \sigma \\
 &= 0.167859 \times 48.58275 \\
 &= 8.155
 \end{aligned}$$

$$\begin{aligned}
 \text{and, Mode} &= \text{Mean} - d \\
 &= 297.608 - 8.155 \\
 &= 289.453
 \end{aligned}$$

Obviously, the sign of d will determine the direction of the location of the mode with respect to the mean, and the sign of d depends upon the sign of x .

x is the mathematical measure of skewness and the distribution is said to be positively skew (or, in everyday language, skew to the right) when x is positive and negatively skew (skew to the left) when x is negative.

Probable Error of the Mode.—Since the mode is located with respect to the mean by means of d , the probable error of the mode is the probable error of d .

$$\begin{aligned}
 PE_d &= 0.67449 \times \sqrt{\frac{3}{2N}} \times \sigma \\
 &= 0.67449 \times \sqrt{\frac{3}{796}} \times 48.58275 \\
 &= 0.67449 \times 0.06139 \times 48.58275 \\
 &= 2.012
 \end{aligned}$$

and the mode is $289.5 \approx 2.01$.

The Coefficient of Variation.—The standard deviation is a measure of spread in terms of concrete units—grams of weight, inches of stature, etc. The coefficient of variation is a measure of dispersion in terms of proportion to the mean.

$$\begin{aligned}
 \text{C.V.} &= \frac{100 \times \sigma}{\text{mean}} \\
 &= \frac{100 \times 48.58275}{297.608} \\
 &= 16.324
 \end{aligned}$$

Probable Error of the Coefficient of Variation.

$$\begin{aligned}
 PE_{CV} &= 0.67449 \times \frac{\text{C.V.}}{\sqrt{2N}} \times \left[1 + \left[2 \times \left\{ \frac{\text{C.V.}}{100} \right\}^2 \right] \right]^{\frac{1}{2}} \\
 &= 0.67449 \times \frac{16.324}{\sqrt{796}} \times \left[1 + \left[2 \times \left\{ \frac{16.324}{100} \right\}^2 \right] \right]^{\frac{1}{2}} \\
 &= 0.67449 \times \frac{16.324}{28.213} \times \sqrt{1 + (2 \times 0.02665)} \\
 &= 0.67449 \times 0.57860 \times \sqrt{1.0533} \\
 &= 0.67449 \times 0.57860 \times 1.0263 \\
 &= 0.401
 \end{aligned}$$

and the coefficient of variation is $16.324 \approx 0.401$.

CHAPTER XLVI

CORRELATION

WITH proper reservations regarding those coincidental relationships that are occasionally encountered, correlation is the term used by statisticians two, or more, series or

Elderton says: "We at the older a bachelor the less likely he is to marry and have children, that a man marrying late in life usually takes a wife who is older than the wife of a man marrying early, . . . all these statements express in different words the fact that there is some causal relationship, or correlation, between the height of a man and the length of his legs (and) between the ages of husband and wife. The statements are, however, in general terms; they do not help us to decide whether one relationship is closer than another; they do not supply any scale of correlation."

When two things increase together, as the ages (at marriage) of husband and wife, the correlation is *positive*. But, when one thing increases as the other decreases, as the age of bachelors at marriage and the number of children born from the marriage, the correlation is *negative*.

The Measurement of Correlation.—For reasons that do not concern us in a practical manual of procedure, correlation is measured by a scale that starts at 0, where there is no correlation, and ends at 1, where there is perfect correlation. With perfect *positive* correlation, $r = +1$; with perfect *negative* correlation, $r = -1$.

SIMPLE CORRELATION

The relationship between two variables is determined by simple correlation. In Table 99, is set up, in a correlation table, two frequency distributions, viz., (1) the body weight of 398 of the Indian pigeons (the same as Table 98) and (2) the heart-weight of these same pigeons. The question is: *Is there any relationship between the body-weight and heart-weight in healthy Indian pigeons?*

The preliminary computations necessary for the calculation of the coefficient of correlation are made a part of the table. In column 1 are placed the total frequencies in the distribution of body-weight, and in line 7 are the corresponding totals in the distribution of heart-weight. The arbitrary axis of the body-weight distribution was placed at 288.5 gm. (the mid-point of the 276 to 300 class), and working units of 25 gm. each, in each direction from this arbitrary axis, are recorded in column 2. The corresponding computations for the heart-weight distribution are recorded in line 8, where the arbitrary axis was placed at 3.125 gm. (the mid-point of the 3.00 to 3.24 class interval). In column 3, the frequencies of each by the
on the
-weight
several

frequencies multiplied by the squares of the respective deviations.

TABLE 99 — A CORRELATION TABLE, WITH THE COMPUTATIONS
Correlation of Heart-weight and Body-weight, 398 Healthy Indian Pigeons

	Heart-weight, in grams												(1)	(2)	(3)	(4)	(5)	(6)
	1 75-1 99	2 00-2 24	2 25-2 49	2 50-2 74	2 75-2 99	3 00-3 24	3 25-3 49	3 50-3 74	3 75-3 99	4 00-4 24	4 25-4 49	4 50-4 74						
175-200	2		1										3	-1	-12	48	-13	52
201-225	3	9	2	7	4								25	-3	-75	225	-75	225
226-250	1	5	12	11	4				1				45	-2	-90	180	-91	182
251-275	2	5	9	10	17	8	5	3	1				60	-1	-60	60	-50	80
276-300		2	6	12	16	22	18	3	3	2			84	0	0	0	-25	0
301-325			1	4	10	16	22	7	8	1	1		76	1	76	76	42	42
326-350				1	9	8	7	6	7	2			42	2	84	168	41	82
351-375				1		7	9	10	11	2	1		11	3	123	369	113	339
376-400						1	1	4	3	4			10	4	61	256	37	148
401-425						1			1				2	5	10	50	3	15
426-450										1	2		3	6	18	108	14	84
451-475										1			1	7	7	49	3	21
(7) Z_x	8	21	31	16	73	60	63	31	35	21	5	1	398		145	1,589	-31	1,270
(8) x	-5	-1	-3	-2	-1	0	1	2	3	4	5	6						
(9) Z_{xy}	-40	-51	-91	-92	-73	0	63	64	105	81	25	6	-31					
(10) Z_{xy}^2	200	376	270	184	73	0	63	136	316	456	125	46	2,083					

Body-weight, in grams											
-----------------------	--	--	--	--	--	--	--	--	--	--	--

In column 5 is recorded the sum of the deviations of heart-weight from the arbitrary axis of heart-weight by deviations from the arbitrary axis of body-weight. For example,

$$(2 \times -5) + (1 \times -3) = -13$$

$$(3 \times -5) + (9 \times -4) + (2 \times -3) + (7 \times -2) + (4 \times -1) = -75, \text{ etc.}$$

In column 6, the values in column 5 have been multiplied by the corresponding values in column 2.

There are two important checks of the correctness of the arithmetic thus far: (a) Column 1 and line 7 must have the same total; (b) the sum of column 5 must equal the sum of line 9.

Moments About the Arbitrary Axes:

$$\begin{aligned}
v_{1x} &= \frac{\Sigma(Z_x x)}{\Sigma Z_x} = \frac{-31}{398} = -0.077859 \\
v_{2x} &= \frac{\Sigma(Z_x x^2)}{\Sigma Z_x} = \frac{2.083}{398} = 5.233668 \\
v_{1y} &= \frac{\Sigma(Z_y y)}{\Sigma Z_y} = \frac{145}{398} = 0.364322 \\
v_{2y} &= \frac{\Sigma(Z_y y^2)}{\Sigma Z_y} = \frac{1.589}{398} = 3.992462
\end{aligned}$$

Moments About the Means:

$$\begin{aligned}
\phi_{1x} &= v_{1x} - v_{1x}^2 = 5.233668 - (-0.077859)^2 \\
&= 5.227601 \\
\phi_{2y} &= v_{2y} - v_{1y}^2 = 3.992462 - (0.364322)^2 \\
&= 3.859731
\end{aligned}$$

If meticulous precision is desired, Sheppard's corrections may be applied to the second moments about the means. It will be very rare that this refinement will significantly affect the final result. In this case, correcting the second moments, $r = 0.730$. The difference is well within one probable error. We shall not bother with the corrections.

The Standard Deviation:

$$\begin{aligned}
\sigma_x &= \sqrt{\phi_{2x}} = \sqrt{5.227601} = 2.286395 \\
\sigma_y &= \sqrt{\phi_{2y}} = \sqrt{3.859731} = 1.964620
\end{aligned}$$

The Coefficient of Correlation:

$$\begin{aligned}
r &= \left[\frac{\Sigma(x y)}{N} - (v_{1x} \times v_{1y}) \right] \times \frac{1}{\sigma_x \times \sigma_y} \\
&= \left[\frac{1.270}{398} - (-0.077859 \times 0.364322) \right] \times \frac{1}{2.286395 \times 1.964620} \\
&= (3.190955 + 0.028377) \times \frac{1}{4.491897} \\
&= 3.219332 \div 4.491897 \\
&= 0.716693
\end{aligned}$$

Probable Error of Correlation Coefficient.—The significance of r is tested from two points of view, viz., (a) Is the correlation significant; i. e., is r significantly different from 0? (b) Is r_{xy} significantly different from r_{ab} ; i. e., is the correlation between x and y significantly different from the correlation between two other variables a and b ?

When tested against zero,

$$\begin{aligned}
PE_{r,0} &= \frac{0.67449}{\sqrt{N}} = \frac{0.67449}{\sqrt{398}} \\
&= \frac{0.67449}{19.9499} = 0.034
\end{aligned}$$

When tested against another coefficient of correlation,

$$\begin{aligned}
PE_{r,r} &= \frac{0.67449}{\sqrt{N}} \times (1 - r) \\
&= 0.033899 \times (1 - 0.716693) \\
&= 0.009399 \times (1 - 0.517629) \\
&= 0.002399 \times 0.482371 \\
&= 0.0012
\end{aligned}$$

The probable error of r , when determined by the foregoing formulas, is relatively accurate only when N is 25, or more. For smaller values of N , these formulas will be found to be unreliable.

LINEAR REGRESSION

The coefficient of correlation (r) is reliable *only when regression is linear*. What, then, is linear regression?

In Table 99, let us find the means of the arrays of body-weight by class intervals of heart-weight; thus:—

175 - 199 array:	2	×	188.5	=	377.0
	3	×	213.5	=	640.5
	1	×	238.5	=	238.5
	2	×	263.5	=	527.0
	8			=	1,783.0
					222.9

and so on for each of the arrays. Now, we shall follow a similar process for each of the arrays of heart-weight by class intervals of body-weight. These means are plotted in Figure 102, the crosses being the means of the body-weight and the circles the means of the heart-weight arrays.

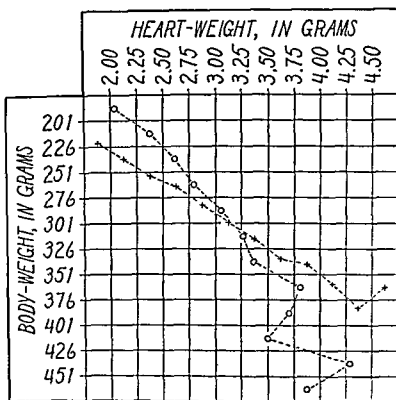


FIG. 102.—Means of arrays of heart-weight and of body-weight.

We mean, by regression, the change in the values of one variable as the values of the other variable increase or decrease. If this relationship between the values of the variable is one of arithmetic progression—that

is to say, if these values plot in a straight line—then the regression is linear.¹

It is apparent at a glance that, in Figure 102, the regression of body-weight upon heart-weight is linear. The same is true of the regression of heart-weight upon body-weight, although perhaps not so apparent to the beginner. The irregularities occur in the small tail arrays where the probable errors of the mean values are very great.

Now, if a straight line ($y = bx$) were fitted to the crosses of the mean body-weight values, we would find that

$$b = 14.48$$

which is to say that there was a variation of 14.48 gm. of body-weight for each 0.25 gm. of heart-weight. But, a straight line fitted in this fashion does not take into consideration the relative weights of the observed points—it gives the same weight to point 363.5, which is determined by only one observation, as to point 282.7, which is the mean of 73 observations.

So, regression lines are fitted by a formula which does take into consideration the weights of the observations.

The regression coefficient for heart-weight upon body-weight is:

$$\begin{aligned} b_x &= r_{xy} \times \frac{\sigma_x}{\sigma_y} \\ &= 0.716698 \times \frac{2.286395}{1.964620} \\ &= 0.716698 \times 1.163785 \\ &= 0.834082 \end{aligned}$$

and the regression coefficient for body-weight upon heart-weight is:

$$\begin{aligned} b_y &= r_{xy} \times \frac{\sigma_y}{\sigma_x} \\ &= 0.716698 \times \frac{1.964620}{2.286395} \\ &= 0.615341 \end{aligned}$$

Now, these regression coefficients are in terms of working units since the σ 's were in those terms. For heart-weight upon body-weight, to get them in terms of grams of weight, we must multiply by 0.25, the range of the class interval. So,

$$0.834082 \times 0.25 = 0.209$$

which is to say that there was, for every increase of body-weight of 25 gm., an increase in heart-weight of 0.209 gm.

In the case of body-weight,

$$0.615341 \times 25 = 15.396$$

which is to say that, for every increase of heart-weight of 0.25 gm., there was an increase of body-weight of 15.396 gm. If the correlation were perfect ($r = 1.00$), these two results would be the same. Compare this properly weighted result with the slope of the line fitted to the unweighted observa-

¹ For a detailed discussion of regression, especially least-squares regression, or *line of best fit*, the reader is referred to Pearl, *Medical Biometry and Statistics*, 2d ed., 1934, 2nd ed., 1937, London, W. H. Saunders Company; 1930 or to Fisher, *Frequency Curves and Correlation*, 2d ed., London, Charles and E. R. Spon, 1927.

tions, which said that there was an increase of 14.48 gm. of body-weight to each 0.25 gm. increase in heart-weight.

Fitting Regression Lines.—The origin of each regression line is at the mean of the opposite variable. It is therefore necessary to find the means of the two variables. From the preceding chapter,

$$\text{Mean} = \text{Arbitrary axis} + r_1$$

The moments, being in terms of working units, must be converted into terms of grams of weight. So

$$\begin{aligned}\text{Mean}_x &= 3\ 125 + [0.25 \times (-0.077889)] \\ &= 3.125 - 0.019472 \\ &= 3.105528 \\ \text{Mean}_y &= 288.5 + (25 \times 0.364322) \\ &= 297.60805\end{aligned}$$

The formula for the regression of heart-weight upon body-weight is

$$x = b_x \times y$$

and for the regression of body-weight upon heart-weight.

$$y = b_y \times x$$

The point of origin of the lines being at the opposite means, we can fit either by actual values or by deviations.

Fitting by actual values,

TABLE 100 — REGRESSION OF HEART-WEIGHT UPON BODY-WEIGHT

(1) Mid-points of body-weight intervals	(2) Deviations from mean of body-weight by 25-gm intervals y	(3) Computed devia- tions of means of arrays from mean of heart-weight z	(4) Computed values of means of heart-weight arrays $X = (x + 3\ 106)$
188 5	-4 36432	-0 910	2 196
213 5	-3 36432	-0 702	2 404
238 5	-2 36432	-0 493	2 613
263 5	-1 36432	-0 284	2 822
288 5	-0 36432	-0 076	3 030
297 608	0 0	0 000	3 106
313 5	+0 63568	+0 133	3 239
338 5	+1 63568	+0 341	3 447
363 5	+2 63568	+0 550	3 656
388 5	+3 63568	+0 758	3 864
413 5	+4 63568	+0 967	4 073
438 5	+5 63568	+1 175	4 281
463 5	+6 63568	+1 384	4 490

The computations in Table 100 are very simple. First converting b_x into actual grams of heart-weight,

$$\begin{aligned}b_x &= 0.834082 \times 0.25 \\ &= 0.20852\end{aligned}$$

we have

$$\begin{aligned}x &= b_x \times y \\ X_{1.00} &= 0.20852 \times (-4.36432) \\ &= -0.910\end{aligned}$$

and

$$\begin{aligned}X &= -0.910 + 3.106 \\ &= 2.196\end{aligned}$$

and so on, for the other intervals of body-weight.

The corresponding Table 101 of fitting the regression line of body-weight upon heart-weight follows:

TABLE 101.—REGRESSION OF BODY-WEIGHT UPON HEART-WEIGHT			
(1) Mid-points of heart-weight intervals	(2) Deviations from mean of heart-weight by 0.25-gm. intervals x	(3) Computed deviations of means of arrays from mean of body-weight y	(4) Computed values of means of heart-weight arrays $Y = (y + 297.608)$
1 875	-4 924	-73 509	221 799
2 125	-3 924	-60 413	237 195
2 375	-2 924	-45 017	252 591
2 625	-1 924	-29 622	267 986
2 875	-0 924	-14 226	283 382
3 106	0 0	0 000	297 608
3 125	+0 076	+ 1 170	298 778
3 375	+1 076	+16 566	314 174
3 625	+2 076	+31 962	329 570
3 875	+3 076	+47 358	344 966
4 125	+4 076	+62 753	360 361
4 375	+5 076	+78 149	375 757
4 625	+6 076	+93 545	391 153

Figure 103 shows the regression lines (the theoretical values of the means of the arrays) together with the actual, or observed, values of the means of the arrays.

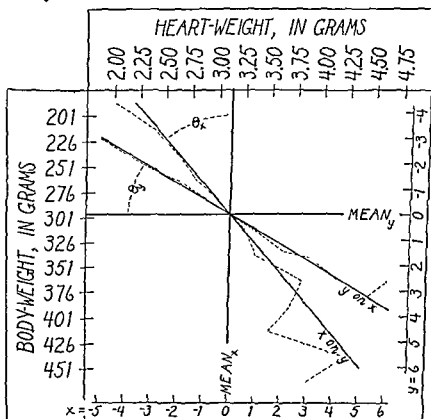


FIG. 103.—Regression lines in the correlation of heart-weight and body-weight, Indian pigeons.

Tests for Linearity of Regression.—For all ordinary work, simple inspection of the plotted regression lines, together with the observed values, is sufficient

The following observation has been made upon the character of the skin lesions in early secondary syphilis in white and colored males:

TABLE 101

Character of lesions	Frequency of occurrence	
	White males	Colored males
Roseola	62	13
Maculo-papular	39	14
Lenticular	19	13
Disoid	1	3
Folliculo-papular	9	28
Folliculo-pustular	0	9
Pustular	0	13
Rupia	5	3
Total cases	135	96

TABLE 105.—COMPUTATIONS IN THE χ^2 COMPARISON OF TWO OBSERVED SAMPLES*

	Roseola	Maculo-papular	Lenticular	Disoid	Folliculo-papular	Folliculo-pustular	Pustular	Rupia	N
White males	(1) 62	39	19	1	9	0	0	5	135
Colored males	(2) 13	14	13	3	28	0	13	3	96
(1) + (2)	(3) 75	53	32	4	37	0	13	8	231
(1) + 135	(4) 0.4593	0.2889	0.1407	0.0074	0.0667	0.0	0.0	0.0370	1.0000
(2) + 96	(5) 0.1354	0.1458	0.1354	0.0313	0.2917	0.0034	0.1354	0.0313	1.0000
(4) - (5)	(6) 0.3239	0.1431	0.0053	-0.0239	-0.2250	-0.0034	-0.1354	0.0037	
Square of (6)	(7) 0.101911	0.020478	0.000028	0.000571	0.050626	0.005798	0.018433	0.000032	
(7) + (3)	(8) 0.001399	0.000386	0.000001	0.000143	0.001368	0.000078	0.001410	0.000004	0.005689

* After Pearl

The question is: Is this difference in distribution significantly greater than might be expected from the operation of chance? The method of applying the χ^2 test to this material is shown in Table 105.

$$\chi^2 = N \times N' \times \sum \left\{ \frac{\left(\frac{f}{N} - \frac{f'}{N'} \right)^2}{\frac{f}{N} + \frac{f'}{N'}} \right\}$$

The sum of line 8 in Table 105 is the expression in the bracket. So

$$\begin{aligned}\chi^2 &= 135 \times 96 \times 0.005689 \\ &= 73.729\end{aligned}$$

and, turning to the table of values of p , we find that, when the number of classes is 8 (n' in the table), and $\chi^2 = 74$, the value of p is less than 0.000001. This is to say that the probability is less than 1 in 1,000,000 that so great a difference in distribution was due to chance. Actually, the probability is less than 1 in 1,000,000,000. It is therefore apparent that, in this sample, there was a very significant difference between the distribution of the types of lesions of secondary syphilis in white and in colored males.

TABLE 106—VALUES OF p FOR CORRESPONDING VALUES OF χ^2 AND n'

		Number of classes — n'							
		8	9	10	11	12	13	14	15
Values of χ^2	12	.1005	.1512	.2133	.2851	.3626	.4457	.5276	.6063
	13	.0721	.1119	.1626	.2237	.2933	.3690	.4478	.5265
	14	.0512	.0818	.1223	.1730	.2330	.3007	.3738	.4497
	15	.0360	.0591	.0909	.1321	.1825	.2414	.3074	.3782
	16	.0251	.0424	.0669	.0996	.1411	.1912	.2491	.3134
	17	.0174	.0301	.0487	.0744	.1079	.1496	.1993	.2562
	18	.0120	.0212	.0352	.0550	.0816	.1157	.1575	.2068
	19	.0082	.0149	.0252	.0403	.0611	.0885	.1231	.1649
	20	.0056	.0103	.0179	.0293	.0453	.0671	.0952	.1301
	30	.0001	.0002	.0004	.0009	.0016	.0028	.0047	.0076
	40	.0000	.0000	.0000	.0000	.0000	.0001	.0001	.0003
	50	.0000	.0000	.0000	.0000	.0000	.0000	.0000	.0000

The Four-fold Table.—One of the most useful of the applications of the chi-square test is that of the measurement of the significance of the difference in occurrence of one event in two samples. This lends itself very well to the evaluation of much experimental data since the two samples can be the

Greenwell, et al., 1914, *Journal of the Royal Army Medical Corps*, 19, 1-10.
typhoid C.
in the British Army. The method, using their data, follows:

TABLE 107

	Did not have typhoid fever	Had typhoid fever	Totals
	a	b	$a + b$
Inoculated	10,322	56	10,378
	c	d	$c + d$
Not inoculated	8,664	272	8,936
	$a + c$	$b + d$	$a + b + c + d$
Totals	18,986	328	19,314

$$\begin{aligned}
 \chi^2 &= \frac{[(a \times d) - (b \times c)]^2 \times N}{(a + b) \times (c + d) \times (a + c) \times (b + d)} \\
 &= \frac{[(10,322 \times 272) - (56 \times 8,664)]^2 \times 19,314}{10,378 \times 8,936 \times 18,956 \times 328} \\
 &= \frac{(2,607,584 - 485,184)^2 \times 19,314}{577,516,167,441,664} \\
 &= \frac{(2,322,400)^2 \times 19,314}{577,516,167,441,664} \\
 &= \frac{5,393,541,760,000 \times 19,314}{577,516,167,441,664} \\
 &= \frac{104,170,865,552,640,000}{577,516,167,441,664} \\
 &= 180.38
 \end{aligned}$$

For the value of p in this application of the chi-square test, we turn to Table 108, which gives the value of p for the several values of $\chi \cdot \left(\chi = \frac{x}{\sigma}\right)$.

$$\chi^2 = 180.38$$

$$\chi = \sqrt{180.38} = 13.4$$

TABLE 108.—VALUES OF p FOR DEVIATIONS MEASURED IN TERMS OF THE STANDARD DEVIATION (σ)

Deviation \sim standard deviation $\frac{x}{\sigma}$	Values of p —the probable occurrence of a deviation as great or greater
0.67449	0.5
1.0	0.3173
1.5	0.1336
2.0	0.0455
2.5	0.0124
3.0	0.0027
3.5	0.000465
4.0	0.0000634
5.0	0.000000573
6.0	0.000000002
7.0	0.000000000026

We find this value completely out of the range of the table, since, when $\chi = 7.00$, $p = 0.000000000026$, or odds of 400,000,000,000 to 1 that no difference as great as this would occur from the mere operation of chance. Thus, the difference in incidence of typhoid fever among inoculated and uninoculated British soldiers was decidedly significant.

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reduced or lost depending on the oxygen consumption of the bacteria present. The reduction time and the quantitative bacterial content of the milk as measured by the colony count have been empirically correlated.

1. **Methylene Blue Reagent.**—Formerly methylene blue was used in the form of methylene blue chloride. This is difficult to purify and standardize and recommended procedure now uses methylene blue thiocyanate which is readily prepared in a state of practical purity and is therefore reproducible. Methylene blue chloride and methylene blue thiocyanate in equal concentrations have identical reduction values in the test. Methylene blue thiocyanate tablets should be used which have been certified by the Commission on Standardization of Biological Stains. Dissolve 1 tablet in 200 cc. of sterile or freshly boiled distilled water observing that the volume of the solution at room temperature is 200 cc. Solution is complete when 1 tablet is allowed to stand in 200 cc. of water overnight. The solution is prepared each week and kept in amber glass bottles in the dark.

2. **Technic.**—Measure 10 cc. of milk into a thick-walled test tube, fitted with a rubber or cork stopper, and add 1 cc. of the certified methylene blue solution. If the blue color is not evenly distributed, invert the corked tube and mix uniformly. Place in a water bath and heat to 37° C., which temperature should be maintained until the test is completed. The tubes should be observed at fifteen-minute intervals, and the end point (disappearance of the blue color) recorded.

3. **Interpretation of Results.**—Within the limits of one and ten hours, any classification of milk based on the methylene blue reduction test is necessarily an arbitrary one. A herd milk that reduces in two hours or less undoubtedly has a high bacterial content. One that requires eight hours for reduction probably contains comparatively few bacteria other than those in the milk at the time of its withdrawal from the udder. The following classification is presented merely as a possible guide. This classification is not intended to carry with it the inference that all milk that decolorizes in less than eight hours is unacceptable for use as market milk.

Class 1.

Class 2.

Class 3.

Class 4.

IV. Sediment Test.—If it is desirable to determine the amount of insoluble sediment in milk, this may be done with the Wisconsin sediment tester (Lorenz Model Company, Madison, Wisconsin). Pint samples of the milk are strained through the special cotton discs which are placed over the opening in the tester. The process may be hastened by warming the milk or using air pressure. After filtering, the "sediment score" or "cleanliness rating" of the milk is determined by comparing the disc with a set of standards ranging from "clean" to "very dirty" milk. Such standards may be secured through the office of the American Public Health Association, 1790 Broadway, New York City.

CREAM

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Mix sample; weigh 1 gm. aseptically into a sterile butter boat or directly into a dilution bottle. (b) *Dilutions Used*.—The allowable bacterial content of cream is greater than that for milk. In order, therefore, to obtain plates for counting which will show the proper number of colonies the dilutions of the cream prepared should be carried one or two steps farther than is done for milk.

ICE CREAM

In the bacteriological examination of ice cream follow the same general methods used for milk. The samples may be conveniently collected in sterile wide-mouthed containers of 125 cc. capacity, fitted with glass stoppers or metal caps. At least 50 cc. of the sample are required. With a sterile, metal spoon remove a portion of the outer surface of the ice cream and use for examination. Sample homogenizer, infrozen

ation outlined under water and milk examination should be used, and if desirable the results may be confirmed by the direct microscopic count method. There are two methods of preparing the dilutions: (1) volumetric, and (2) gravimetric.

1. *Volumetric*.—In order to reduce the percentage of error when using materials of high viscosity it is necessary to use large amounts in making the first dilution. Frozen ice cream should be melted by placing the container in a water bath at 45° C. for about fifteen minutes. Higher temperatures or longer periods of heating are undesirable. Place 11 cc. of the melted material in 99 cc. of sterile water for the first dilutions (1 to 10) and with this make further suitable dilutions and prepare agar pour plates.

2. *Gravimetric*.—Heat the sample to a suitable consistency in a water bath (43° to 45° C.) or until it has attained a temperature of 10° C. Prepare a sterile test tube containing a butter boat (Mojonnier Bros., Chicago) or a similar apparatus of a size that will be admitted to the neck of the dilution flask. Remove the cotton plug from the test tube and slide the boat to a point about $\frac{3}{4}$ inch beyond the end of the tube. Place the tube and boat on the pan of a balance and weigh to the second decimal place. With a wide-mouthed pipet place 1 gm. of the sample on the boat. Slide the boat containing the sample through the neck of a dilution flask containing 99 cc. of sterile water. Make further dilutions of this 1 to 100 suspension, and prepare agar pour plates. Incubate the plates for forty-eight hours at 32° or 37° C. and report the results as the "standard plate count."

DETECTION OF PATHOGENIC ORGANISMS

In the routine bacteriological examination no attempt is made to identify the species of bacteria present in milk. However it is often desirable to make special examinations to discover whether fecal contamination has occurred. Fresh milk may, without prejudice, be found to contain small numbers of coliform bacteria but there should be none in pasteurized milk and if found these organisms would indicate either: (a) a defect in the pasteurizing process, or (b) pollution of the milk after pasteurization.

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Pathogenic bacteria may also be present for milk can transmit tuberculosis, typhoid fever, septic sore throat, scarlet fever, diphtheria and undulant fevers. Therefore in the examination of milk it may be necessary to look for the organisms responsible for such diseases.

I. Coliform Bacteria.—The presence of these organisms which may be taken as an indication of fecal pollution, is determined by methods similar to those recommended for the examination of water.

II. Tubercle Bacilli.—Centrifugalize 30 cc. of the milk at high speed and collect the lower 10 cc. with the sediment. Inject 3 cc. amounts of this subcutaneously into each of two guinea pigs and a rabbit. Collect 4 cc. of the cream, thin with a little sterile water and inoculate like portions pigs and a rabbit. As some of the other "butter" bacillus,* may also cause lesions, from those due to tubercle bacilli to avoid

error. This may be done as follows: (a) Inoculate glycerol agar with material from the lesions and incubate at 37° C. The confusing acid-fast bacteria usually form colonies within a few days while tubercle bacilli do not. (b) When ready to test the animals, inoculate 2 cc. of old tuberculin into each. The tuberculous animals will die within a day. Also if virulent tubercle bacilli are present the typical lesions should be easily recognized.

III. Typhoid Bacilli.—Milk may be examined for *Eberthella typhosa* or other related organisms as follows: (a) Centrifugalize a portion of the milk, combine the cream and sediment and with this inoculate eosin-methylene blue agar plates, and nutrient broth (see page 478); (b) Incubate the remainder of the specimen and at intervals make transfers to the media indicated above. Transfer selected colonies to fresh media and identify the organisms as outlined under *Eberthella*. The same technic may be used to isolate *Salmonella* from milk and the possibility that *Shigella* may be present must not be overlooked. For the isolation of these two groups the *Salmonella-Shigella* agar should be used instead of the Wilson-Blair medium.

IV. Streptococci.—Hemolytic streptococci are found in the udders and milk of cows even when kept under the best conditions, but the alpha is the beta type. It is difficult to dis- the saprophytic streptococci and those It has been suggested that the organisms

responsible for milk-borne human infections are probably always derived from cows in which the udder has been infected from human sources, or from milk contaminated by human beings. The streptococci causing septic sore throat and scarlet fever are transmissible through milk. They may be isolated for study by the technic outlined below for diphtheria bacilli and identified as indicated under Streptococci.

V. Diphtheria Bacilli.—Contamination of milk with diphtheria bacilli can usually be traced to some human source. In examining suspected samples for *C. diphtheria*, centrifugalize the milk and use a mixture of the cream and sediment for the inoculation of blood agar plates. Examine stained films made from individual colonies, and transfer typical growths to fresh media for isolation and identification.

* *Mycobacterium lacticola* now includes a variety of incompletely described "species" from the environment, including butter.

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VI. *Brucella*.—Centrifugalize the milk and use a mixture of cream and sediment for inoculation on plates of liver infusion agar and tryptose agar containing crystal violet in a final dilution of 1 to 700,000 (the dye inhibits the growth of many Gram-positive organisms commonly found in milk). Incubate at 37° C. in an atmosphere containing 10 per cent CO₂ (the aerobic species *Br. suis* and *Br. melitensis* will not be inhibited by the CO₂, *Br. abortus* requires it) for five days. It is difficult to isolate these organisms from milk if streptococci are present. Inoculate 5 cc. of the centrifugalized cream intraperitoneally into each of two 250-gm guinea pigs and inoculate 5 cc. of sediment suspension into each of two other guinea pigs. If the animals survive six weeks they should be sacrificed and the liver and spleen examined for lesions. After isolation of the organisms from the cultures or animals identify by the method outlined under *Brucella*. It is also possible to show the presence of agglutinins for the undulant fever organisms in the milk of infected animals.

CHAPTER XXIX

EXAMINATION OF SHELLFISH

By LELAND W. PARR

As oysters and other shellfish have been incriminated as the source of epidemics of typhoid and other enteric infections, bacteriological examinations may at times be desirable in determining their sanitary condition. A committee of the laboratory section of the American Public Health Association has just reported on the subject under the title "Bacteriological Examination of Shellfish and Shellfish Waters" (American Journal of Public Health, 1943, 33, pp. 582-591 [May]). A new "Standard Methods" will shortly be published.

The term "shellfish" as the committee uses it refers to oysters, soft-shell clams, quahaugs and mussels. The Crustacea—shrimp, crabs, lobsters, etc.—are not included as "shellfish," and there is for this group of foodstuffs no official standard procedure. This does not mean there are no sanitary problems or controls in connection with these foodstuffs for such "hand-picked" material is of necessity subject to pollution and without due supervision may be processed under unsanitary conditions.

From biological studies of shellfish and the special problems are involved. Under certain conditions it is possible to obtain not only a quantitative estimate of the coliform bacteria but of the relative proportion of *Escherichia coli* present."

for *Escherichia coli*.

The coliform group is considered, in this connection, "as including all organisms which, upon transfer from a positive presumptive test (gas positive in lactose broth), grow in lactose broth containing 0.0013% bile (brilliant green-lactose broth). Any organism considered to be any member of this group which in pure culture fails to grow in Koser's citrate medium within twenty-four hours, or which shows

that in these examinations the aim is to arrive at an idea of the number of coliform bacteria present and whether citrate-negative, Eijkman-positive *Escherichia coli* is the species represented or to what degree it is present. (*Esch. coli*, so characterized is presumed to be evidence of fecal pollution.) Ten oysters are selected, cleaned and opened. Each is cut into ten equal pieces and combined with the shell liquor. This is shaken thoroughly with glass beads and an equal volume of sterile distilled water added with additional shaking. The material is then allowed to settle for

two minutes and plain lactose broth fermentation tubes inoculated with the supernatant (15 tubes: 5 with 2 cc., 5 with 1 cc. of a 1 in 10 dilution, 5 with 1 cc. of 1 to 100 dilution). Calculations of the "most probable number" of coliform bacteria present are made from the fermentation results observed for not more than forty-eight hours. (Formerly the results were expressed in terms of a "Score" calculated from the test results.)

Lactose broth tubes showing gas are "confirmed" by transfer to brilliant green-lactose-bile broth and the diagnosis of *Escherichia coli* is established by plating on eosin-methylene blue agar and testing a pure culture for growth in Koser's citrate medium and in modified Eijkman lactose medium at 45.5° C.

Shellfish-growing waters may be examined by regular water analysis procedure provided that the method used provides the proper information—"most probable number" of coliform bacteria per 100 cc. of sample and allows for the demonstration of *Escherichia coli*. The examination of "shucked" oysters is made in an analogous manner to that for "shell stock." Inasmuch as Hoskin's tables of Most Probable Numbers are used in calculating results in both shellfish and water analysis as at present interpreted these tables are included here. (See Tables 74 and 75.)

TABLE 74.—MOST PROBABLE NUMBERS (HOSKINS)

(Most probable numbers per 100 cc. of sample, planting various portions in not more than 3 dilutions.)

Number of positive tubes in dilutions			Combinations of portions planted in cc.			Number of positive tubes in dilutions			Combinations of portions planted in cc.				
Low	Mid	High	2-10	1-10	1-50	Low	Mid	High	5-10	5-10	5-10	5-100	5-100
			2-1	5-1	5-10				(2)	1-1	1-1	1-10	5-10
0	0	1	2	0.1	1-1	0	0	1					
0	0	2	4	5	6	0	0	0					
0	0	3	9	0	1.0	0	1	0					
0	1	0	4	6	1.0	0	2	0		2 0	2 0	0 18	0 18
0	1	1	9	2	2 1	0	3	0			2 0	0 19	0 37
0	1	2	14	0		0	4	0					0 56
0	2	0	9	4	2 2	0	1	1					0 75
0	2	1	14	0	3 3	1	0	0	2 2	2 2	4 0	0 38	0 20
0	2	2	19	0		1	0	1			4 4	0 40	
0	3	0		22	3 5	1	1	0			4 4	0 42	0 41
0	3	1		30	4 7	1	2	0					0 72
0	4	0		31	5 0	1	3	0					0 84
0	4	1		32	6 4	1	4	0					1 1
0	5	0		40	6 8	1	1	1			6 7	0 63	
0	5	1		49	8 3	2	0	0	5 1	5 0	5 0	0 44	0 45
1	0	0	6	0	11	2	0	1			7 5	0 64	
1	0	1	12	0	2 9	3	0	0			7 6	0 71	0 69
1	1	0	13	0	3 1	3	1	0					0 94
1	1	1	20	0	4 9	3	2	0					1 2
1	1	2	28	0		3	3	0					1 5
1	2	0	21	0	5 5	3	0	0	9 2	8 9	10 0	0 57	0 72
1	2	1	29	0	7 9	3	0	1			12 0	1 1	
1	2	2	37	0		3	1	0			12 0	1 1	1 1
1	3	0		49	9 0	3	2	0					1 4
1	3	1		120	12 0	3	3	0					1 8
1	4	0		150	15 0	4	4	0					2 1
1	4	1		210	21 0	4	1	1			16 0	1 5	1 2
1	5	0		340	39 0	4	0	0	16 1	15 0	15 0	1 7	
2	0	0				4	0	1			20 0	1 7	1 7
2	0	1	23	0		4	1	0			21 0	1 8	2 2
2	1	0	50	0		4	2	0					2 6
2	1	1	65	0		4	3	0					2 8
2	1	2	82	0		4	4	0					3 1
2	2	0	130	0		4	1	1					
2	2	1	210	0		5	0	0			27 0	2 4	2 4
2	2	2	240	0		5	0	1			28 0	3 1	
2	3	0	340	0		5	1	0			29 0	4 7	3 5
						5	2	0					4 8
						5	3	0					5 3
						5	4	0					6 0

as much medium as the amount of water to be tested. When required to examine larger amounts than 10 cc. as many tubes as necessary shall be inoculated with 10 cc. each.

The portions of the water sample used for inoculating the lactose broth fermentation tubes will necessarily vary in size with the character of the water under examination. When examining water for evidence of conformance to the United States Public Health Service standards inoculate a series of five lactose broth fermentation tubes, each with a 10 cc. portion of the sample (see page 547). When examining water of questionably potable quality use appropriate gradations which may go as low as 0.01 cc. or even involve dilution. In general, the largest portions selected for sampling should result in gas production in all or most of the broth tubes into which they are inoculated whereas the smallest portions should produce no gas in all or the majority of the broth tubes into which they are planted. In such analyses there are usually more than two quantitative portions, *e. g.*, 10 cc., 1 cc., 0.1 cc. and 0.01 cc.

(b) *Incubation and Reading.*—Incubate these tubes at 37° C. for forty-eight hours. Examine each tube at twenty-four and forty-eight hours and record gas formation. The records should be such as to distinguish between:

- (1) Absence of gas formation.
- (2) Formation of gas occupying less than 10 per cent of the inverted vial.
- (3) Formation of gas occupying 10 per cent or more of the inverted vial.

More detailed records of the amount of gas formed, though desirable for the purpose of study, are not necessary for carrying out the standard tests prescribed.

(c) *Positive Presumptive Test.*—Formation within twenty-four hours of gas occupying 10 per cent or more of the inverted vial in the fermentation tube constitutes a positive presumptive test.

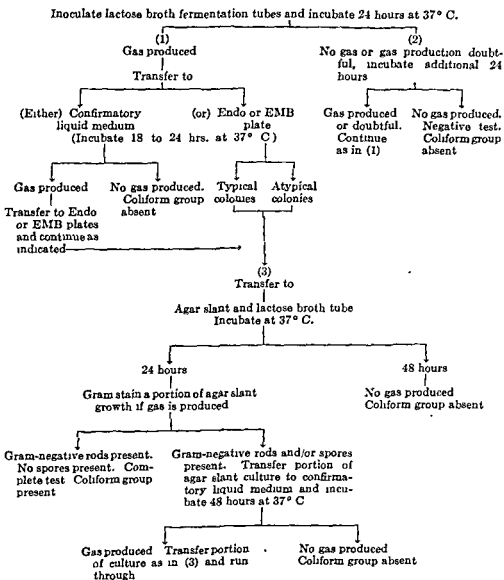
(d) *Doubtful Test.*—If no gas is formed in twenty-four hours, or if the gas formed is less than 10 per cent, the incubation shall be continued to forty-eight hours. The presence of gas in any amount in such a tube at forty-eight hours constitutes a doubtful test, which in all cases requires confirmation.

(e) *Negative Test.*—The absence of gas formation after forty-eight hours incubation constitutes a negative test. An arbitrary limit of forty-eight hours observation doubtless excludes occasional members of the coliform group which form gas slowly. This is a problem as yet unsolved. In practice since practically all fecal contamination is communal in origin the failure to reveal "atypical coliform" or "paracolon" bacteria is inconsequential since in such increment of contamination there will undoubtedly be also present typical coliform bacteria which will produce gas and thus reveal the contamination. In theory fecal material coming from a single individual whose flora was "atypical" could be present and be completely overlooked. In such a case the count at 37° C. if made, should betray the situation.

3. *Confirmed Test.*—The use of Endo's or eosin-methylene blue plates or of brilliant green-lactose-bile broth provides two methods for carrying further the analysis of the water sample for coliform bacteria. (a) *Preparation of Plates.*—Make one or more Endo or eosin-methylene blue plates

(d) *Completed Test for Brilliant Green Confirmation.*—If it is desired to complete the test when confirmation has been made using brilliant green-bile-lactose broth an Endo or eosin-methylene blue plate is streaked from the brilliant green Positive Confirmation. Colonies developing, if any, are subjected to the routine indicated in (a), (b) and (c) immediately above. Table 76 presents in outline form the procedures involved in the "Completed Test."

TABLE 76.—COMPLETED TEST



5. *Selection of Coliform Tests.*—When the laboratory worker elects to apply either the Presumptive, the Confirmed or the Completed Test for the coliform group he should be guided by the following basic considerations:

(a) *Completed.*—Apply to the smallest gas-forming portion or portions of:

(1) Any sample of drinking water upon which sufficient data are not available to justify the application of the Confirmed Test;

(2) Any sample of drinking water upon which the previous records indicate the inapplicability of the Confirmed Test;

(3) Any sample of drinking water which is being examined with reference to the U. S. Federal Security Agency Standard.

(b) *Confirmed Test*.—Apply to the smallest gas-forming portion or to all portions showing gas, from:

(1) Any sample of raw water, water in process of purification, or water prepared for human consumption where a sufficient number of prior examinations have established a satisfactory correlation of the Confirmed Test with the Completed Test;

(2) Miscellaneous samples from any other source when it is known that the Presumptive Test is too broadly inclusive.

(c) *Presumptive Test*.—Apply:

(1) In the examination of sewage, sewage effluents, or water showing relatively high pollution, where fitness for use as drinking water is not under consideration, and

(2) In the routine examination of raw waters in purification plants, provided that records indicate that the Presumptive Test is not too inclusive for the production of data statistically comparable to that obtained upon the finished water.

6. *Quantitative Estimate of the Coliform Group*.—In order that tests for the coliform group may have quantitative significance the following general principles and rules should be observed: (a) Ordinarily not less than three portions of each sample should be tested, the portions being even decimal multiples or fractions of 1 cc., e. g., 10, 1, 0.1, the selection of the volumes of the samples chosen conforming to the suggestions made above under "Presumptive Test." The quantitative value of a series of tests is lost unless all or a majority of the smallest amounts tested have given negative results. (b) In recording a single test, it is preferable to record results as observed, indicating the amounts tested and the result in each, rather than to attempt expression of the result in numbers of organisms per cc. In summarizing the results of a series of tests, however, it is desirable, for the sake of simplicity, to express the results in terms of the number of coliform organisms per cc. or per 100 cc. There are two methods applicable to this situation.

(a) *Simple Quantitative Coliform Calculation*.—The number of organisms per cc. may be expressed as the reciprocal of the smallest portion (expressed in cc.) giving a positive result. For example, the result: 1 cc. plus, 0.1 cc. plus, 0.01 cc. negative, would be recorded as 10 per cc. An exception should be made in the case where a negative result is obtained in an amount larger than the smallest portion giving a positive result; for example, in a result such as: 10 cc. plus, 1 cc. minus, 0.1 cc. plus. In such case, the result should be recorded as indicating a number of coliform organisms per cc. equal to the reciprocal of the portion next larger than the smallest one giving a positive test, this being a more probable result. Where tests are made in amounts larger than 1 cc. giving average results less than one organism per cc. it is more convenient to express results per 100 cc. In order that results as reported may be checked and carefully evaluated, it is necessary that the report should show not only the average number of organisms per cc., but also the number of samples examined; and, for

each volume tested, the total number of tests made, and the number (or per cent) positive. A sample test shown in Table 77 illustrates the method.

TABLE 77.—RESULTS OF TESTS IN AMOUNTS DESIGNATED. INDICATED NUMBER OF ORGANISMS OF THE COLIFORM GROUP

10 cc.	1 cc.	0.1 cc.	0.01 cc.	Per cc.	Per 100 cc.
+	—	—	—	0.1	10
+	+	—	—	1.0	100
+	+	+	—	10.0	1,000
+	+	+	+	100.0	10,000
+	+	—	+	10.0	1,000
Totals (for estimating averages):				121.1	12,110
Average of five tests (adjusted to significant figures):				24.0	2,400

The result obtained by this method is sometimes called the I.N. ("Indicated Number") value.

(b) *Most Probable Number*.—A much more statistically satisfactory method of expressing results is to interpret the results in terms of the "Most Probable Number" of organisms present. The values calculated by Hoskins shown in Tables 74 and 75, are used for this purpose. In these tables where the terms low, mid and high are used at the head of a column they refer to the low, mid or high dilution and indicate respectively the greatest, middle and least portion of the original sample. The most desirable procedure for obtaining a single numerical value for a series of analytical results is to express the results of each analysis in terms of its M.P.N. (Most Probable Number) value and strike an arithmetical average of these values. It is mathematically incorrect to summarize the number of positive tubes in the various dilutions for a number of samples from the same source on the same day or over a period of days and use an arithmetical average of the accumulated figures to determine a single M.P.N. for the series of samples.

7. Interpretation of Coliform Results.—The definition of the coliform group as given in Standard Methods includes organisms of both the so-called fecal and non-fecal types. At the present time, any attempt to evaluate a drinking water on the basis of a distinction between these two types is regarded as unwarranted. In case it is desired to make such a distinction it will be recalled that *Escherichia coli* is indol-positive, methyl-red-positive, Voges-Proskauer negative and citrate negative (Imvic ++--) whereas *Aerobacter aerogenes* is Imvic --++. An extremely wide gamut of intergrading types of coliform bacteria are known to exist. The best recognized of these is *Escherichia freundii* which is Imvic -+-+. Dominant (whether pathogenic or not) groups of bacteria, such as the coliforms, have maintained their position of importance, in part at least,

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and only that on all occasions and in all circumstances. The coliform group still remains our best indicator but the move begun some years ago to put great store by its break-down into species has not made recent headway.

VI. Drinking Water Standards.—The following standards were adopted by the Federal Security Agency, December 3, 1942; for drinking and culinary water supplied by common carriers in interstate commerce.

1. **As to Source and Protection.**—(a) The water supply shall be:

- (1) Obtained from a source free from pollution; or
- (2) Obtained from a source adequately protected by natural agencies from the effects of pollution; or
- (3) Adequately protected by artificial treatment.

(b) The water supply system, including reservoirs, pipe lines, wells, pumping equipment, purification works, distributing reservoirs, mains and service pipes, shall be free from sanitary defects.

2. **As to Physical and Chemical Characteristics.**—The water should be clear, colorless, odorless and pleasant to the taste, and should not contain an excessive amount of soluble mineral substances nor of any chemicals employed in treatment.

3. **As to Bacteriological Quality.**—The bacteriological examinations which have come to be generally recognized as of most value in the sanitary examination of water supplies are:

(a) The count of the total colonies developing from measured portions planted on gelatin plates and incubated for forty-eight hours at 20° C.

(b) A similar count of total colonies developing on agar plates incubated for twenty-four hours at 37° C. (or in some laboratories incubated forty-eight hours at 20° C.).

(c) The quantitative estimation of organisms of the coliform group by applying specific tests to multiple portions of measured volume.

Of these three determinations, the test for organisms of the coliform group is almost universally conceded to be the most significant, because it affords the most nearly specific test for the presence of fecal contamination. The committee has, therefore, agreed, after full consideration, to include only this test in the bacteriological standard recommended, believing that neither the 37° C. nor the 20° C. plate count would add information of sufficient importance to warrant complicating the standard by including them in the required examination. The omission of plate counts from the standard is not to be construed, however, as denying or minimizing their importance in routine examinations made in connection with the control of purification processes. On the contrary, the committee has stated as its opinion that one or both plate counts are of definite value, and has emphasized that it is chiefly in the interest of simplicity that plating methods have been omitted from the standard here proposed.

(d) Of all the standard (10 cc.) portions examined per month in accordance with the procedure specified, not more than 10 per cent shall show the presence of organisms of the coliform group.

(e) Occasionally three or more of the five equal (10 cc.) portions constituting a single standard sample may show the presence of coliform bacteria. This shall not be allowable if it occurs in more than:

(1) Five per cent of the standard samples when 20 or more samples have been examined per month.

(2) One standard sample when less than 20 samples have been examined per month.

Two procedures are suggested in the 1942 revision. In one, as mentioned above, the size of the sample is 10 cc. and the test consists of five portions thereof. In the other, the standard portion of water is 100 cc. and the test consists of five such portions. In any disinfected supply the sample must be freed of any disinfecting agent within twenty minutes of the time of its

collection. It is optional whether 10 cc. or 100 cc. portions be used. The technic of interpretation is slightly different for the 100 cc. procedure which is reasonable in view of the large volume of water under test. In both cases if the water supply fails to meet standards it is provided that daily samples be examined from such an unsatisfactory source until the water is again of satisfactory quality. When this occurs and when waters of unknown quality are being examined simultaneous tests should be made on multiple portions of a geometric series ranging from 10 cc. to 0.1 cc. or less.

The Public Health Service standards provide also that the frequency of sampling and the distribution of the sampling points shall be regulated by the certifying authority after investigation of the source, method of treatment and protection of the water concerned. The standards also prescribe the minimum number of samples to be collected and examined each month which depends upon the population served. Thus when no more than 2500 persons are served by a water supply the minimum number of samples per month is one; where 100,000 are served the minimum is 100; and where five million persons constitute the population at least 500 samples per month must be examined. The laboratories in which examinations are made and the methods used shall be subject to inspection at any time.

SWIMMING POOLS

To ensure the greatest possible freedom from infection among those bathing in swimming pools it is essential that the sterilization procedures used be constantly maintained at a high degree of efficiency. This can be determined by daily bacteriological examinations of the water collected during the period of greatest use of the pool.

An official report of the American Public Health Association (1942) entitled "Recommended Practice for Design, Equipment and Operation of Swimming Pools and Other Public Bathing Places" provides that "when-ever chlorine, calcium hypochlorite, or other chlorine compounds, without the use of ammonia, are used for swimming pool disinfection, the amount of available or excess chlorine in the water at all times when the pool is in use shall not be less than 0.4 p.p.m. or more than 0.6 p.p.m. (parts per million). Whenever chlorine or chlorine compounds are used with ammonia, the amount of available or excess chloramine shall not be less than 0.7 p.p.m. or more than 1.0 p.p.m." Standards for determining chlorine residuals shall be prepared and used according to the recommendations in Standard Methods of Water Analysis (A.P.H.A., 1936). Standardized color discs and comparators may be used. For the elimination of algae the use of copper sulfate in concentration of 3.6 to 4.2 p.p.m. has given satisfactory results.

The report provides that all chemical and bacterial analyses be made in accordance with the procedures recommended in the Standard Methods of Water Analysis in so far as these methods apply to swimming pool waters. Specifically (a) not more than 15 per cent of the samples covering any considerable period of time shall contain more than 200 bacteria per cc. counted on standard nutrient agar after twenty-four hours incubation at 37° C.; and (b) not more than 15 per cent shall show the positive test (confirmed) in any of five 10 cc. portions of water at times when the pool

is in use. All primary fermentation tubes showing gas should be confirmed. The residual chlorine in water samples from pools must be neutralized with sodium thiosulfate (as we have earlier indicated) otherwise the water may be found on analysis to be bacteria-free when in reality at the pool it was perhaps unfit for use.

The part played by the various strains of streptococci in the respiratory diseases and their prevalence in the intestinal, buccal, and nasal discharges make the presence of these organisms in bathing waters very undesirable. At the present time, however, it is not practicable either to adjust the bathing load and treatment so as to eliminate them or to suggest a satisfactory test and minimum standards for their presence.

RAW WATER

Army Regulations (A.R. 40-205) provide that "all water will be considered impure until declared potable by the proper medical officer." In fixed posts water is normally treated. In the field water may be treated by means of mobile or portable filters of the Corps of Engineers, in the water sterilizing bag, or in other suitable container. A good water is not only one potable from bacteriological points of view; it must have desirable chemical and physical properties and treatment is sometimes to effect changes in these regards as well as in bacterial content. Calcium hypochlorite is used with water in the standard 36-gallon canvas water-sterilizing bag provided by the Army or in its absence in other suitable container to effect bacteriological potability. One-half gm. of calcium hypochlorite is required for each 36 gallons of water and the chemical is supplied by the Quartermaster Corps in measured doses in sealed glass tubes. The contents of one tube are dissolved in a cupful of water and the entire contents

the hypochlorite solution.

When large containers are not available an official tube of calcium hypochlorite ($\frac{1}{2}$ gm.) will be dissolved in a cupful of water and placed in an empty canteen which is then filled with water and shaken. This strong solution may then be used to sterilize water in other canteens by adding 1 canteen cap full (approximately $1\frac{1}{2}$ teaspoonfuls) to each canteen full (approximately 1 quart) of water. After thirty minutes the water is bacteriologically potable. When no hypochlorite is available water of unknown purity may be considered safe for drinking after it has been boiled for *one minute*.

SEWAGE

Sewage effluents and grossly polluted water may be examined for organisms of the coliform group or other bacteria by the technic outlined for water analysis except that the specimens must be used in higher dilutions. The total colony count is of importance in the examination of sewage.

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CHAPTER XXXI

ORGANISMS OF UNCERTAIN CLASSIFICATION

By QUENTIN M. GEIMAN

TOXOPLASMA

I. Toxoplasmosis.—Protozoa of uncertain affinities belonging to the genus *Toxoplasma* have been recently recognized as the cause of human disease. Available evidence indicates that these organisms produce: (1) certain types of congenital encephalitis and hydrocephalus *in utero*, during the newborn period, infancy, and early childhood; (2) acute encephalitis in childhood and (3) a spotted fever-like disease associated with atypical pneumonia in adults.² Sabin³ summarizes the described cases of infection and lists 12 cases in infants, 2 in children six and eight years of age, and 3 in adults. The incidence of the disease is entirely unknown, but positive tests for neutralizing antibodies⁴ indicate that the disease is congenital in ted infants and children present or past infection. the transmitters of adult infection, but the possibility of transmission by droplet infection has been suggested.¹

II. Description of Organism.—*Toxoplasma* are minute obligate intracellular parasites (Fig. 43, No. 1), varying in size from $4 \text{ to } 7 \times 2 \text{ to } 4 \mu$. The organisms divide by binary fission and are crescentic in shape with one end pointed and the other end rounded (Fig. 43, No. 3). Distinct cytoplasm and nuclear chromatin makes up the body of the organism, but there are no flagella or visible accessory nuclear structures. Growing forms become oval or round prior to division (Fig. 43, No. 2). They may occur singly, in clusters or aggregates within fixed host cells (Fig. 43, Nos. 4 and 5). Cells of the reticulo-endothelial system, parenchymal cells of lung, liver, adrenals, brain, unstriated or striated muscle and kidney may be parasitized. Infections in the fetus and early infancy produce marked destruction of mucous tissue, but the viscera are primarily invaded in the adult cases. In fact, masses of parasites, ranging up to $51 \times 10 \mu$, have been found in cardiac muscle.¹

The morphology of this parasite is sufficiently characteristic in most cases to differentiate it from other organisms or tissue cells. The absence of a parabasal body in the cytoplasm differentiates it from the aflagellate stages of *Leishmania* and *Trypanosoma cruzi*. Differentiation from *Sarcocystis* and *Encephalitozoön* is more difficult and detailed descriptions of these organisms should be consulted if there is any question about diagnosis.²

III. Diagnosis.—Specific clinical symptoms of toxoplasmosis, although suggestive, are not clarified enough as yet to provide more than presumptive evidence of infection. The following laboratory procedures are necessary in body tissues *toxoplasma* by cultivation (3) demonstration of specific antibodies.

1. **Direct Examination.**—Impression films of suspected tissues or fluids should be air dried and stained with Wright's or Giemsa stain. Since organisms are seldom demonstrable in blood, films should be made from biopsy tissue, sediment of cerebrospinal, pleural and peritoneal fluids,



FIG 43.—*Toxoplasma* ($\times 1100$ except No. 3). 1, *Toxoplasma* in a leukocyte; 2, free and dividing *Toxoplasma* in impression film. 3, typical morphology of free *Toxoplasma* and dividing organisms in leukocyte ($\times 2000$); 4, large aggregate of *Toxoplasma* in section of human heart; 5, small group of organisms in section of human liver. (Photographs 4 and 5 made from slides in the collection of Dr. D. Weisman.)

sputum, and vaginal exudates. To demonstrate the parasite in sections, use Giemsa stain after fixation with Regaud's fluid or eosin methylene blue after Zenker-acetic fixation.² Staining with hematoxylin and phloxin, and hematoxylin and eosin after formalin fixation is also satisfactory.³

2. **Isolation of Organisms.**—Growth of *Toxoplasma* in the developing chick embryo and in a surviving tissue culture medium (minced chick embryo in Tyrode's solution) have been obtained,⁶ but the methods although promising, have not been used for practical diagnosis. The preferred method is to inoculate fresh untreated tissue or fluids into albino mice, guinea pigs, and rabbits, all of which are highly susceptible to the infection. Sabin³ suspends the tissue in Tyrode's or physiological saline solution and inoculates a minimum of 6 mice and 2 guinea pigs—0.03 cc. are injected intracerebrally and 1 cc. intraperitoneally into each mouse and 0.2 cc. intracerebrally and about 5 cc. intraperitoneally into each guinea pig. The mice are observed for a period of a month and the guinea pigs for six weeks. Films and sections of the brain and lungs of animals that become sick or die are examined for *Toxoplasma*. Passage to additional animals may be performed to test pathogenicity by inoculating a pooled suspension of fresh and untreated ground brain and viscera from the infected animals.

3. **Serological Tests.**—Studies on toxoplasmosis have shown that recovery from infection confers immunity to reinfection. The broad host range of this parasite and the apparent unity of species involved is the basis for some differences in the nature of the immune state from host to host. Although our knowledge is far from complete, demonstrations of humoral antibodies in patients with toxoplasmosis and experimental animal infections have provided two tests that are of value for diagnostic purposes, (1) demonstration of neutralizing antibodies,^{5,6} and (2) the complement-fixation test.⁸

The test for neutralizing antibodies is made on the back of a rabbit,⁴ with fresh serum to be tested and a *Toxoplasma* suspension prepared from brains of mice succumbing in four days to a highly virulent strain of the parasite. Suitable dilutions of suspension are made plus 0.15 cc. undiluted

dilution inoculated per square is 0.15 cc.

Lesions caused by *Toxoplasma* begin to appear by the fourth day,

of generalized toxoplasmosis between the ninth and twelfth day. Marked lesions by a serum indicate the presence of *Toxoplasma*. Papers of Sabin^{3,4,7} should be consulted for details of the test.

The complement-fixation test of Warren and Sabin⁸ will provide a result more quickly than the neutralization test and for that reason is more practical. Difficulties in preparing a suitable antigen and discrepancies between the results of the two serological tests indicate that the procedure needs improvement. Thus, in the absence of direct demonstration of parasites, and in the presence of one or more positive immunologic tests, positive animal inoculations are needed to verify diagnosis. Positive serological tests occur as evidence of past as well as present toxoplasma infection. Since adult toxoplasmosis has a close clinical resemblance to that of rickettsial diseases, emphasis must be placed on a careful search for

Toxoplasma in individuals with a spotted fever-like syndrome associated with atypical pneumonia.¹

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BARTONELLA

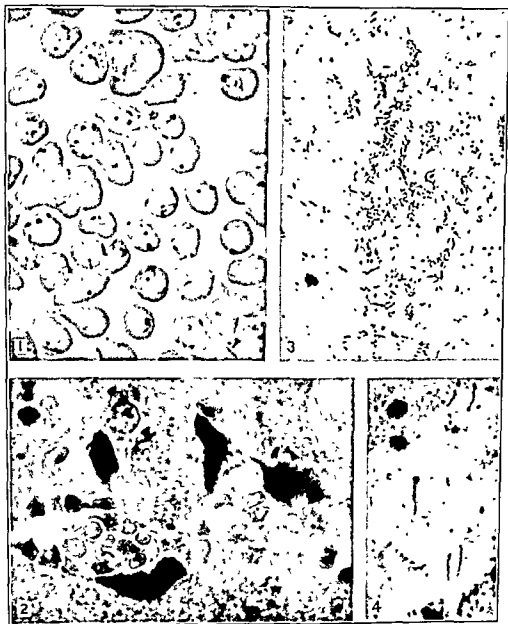
I. **Bartonellosis.**—A disease of humans in Peru, Ecuador, and Colombia, known as Carrion's disease, is caused by an organism called *Bartonella bacilliformis*. Two phases of the disease make up the syndrome: (1) Oroya fever or the stage characterized by a blood stream infection with *B. bacilliformis*, progressive anemia, prostration and a mortality which may reach 40 per cent, and (2) *Verruga peruana* or the eruptive stage characterized by miliary or nodular eruptions containing the parasites, slow convalescence and rare mortality. Asymptomatic carriers with blood stream infections detectable only by culture procedures occur in the endemic regions.

II. **Morphology.**—*B. bacilliformis* is a minute pleomorphic, motile, bacillus-like organism which varies in shape from rounded bodies to straight rods. The organisms may be d in size from 1 to 2 μ and may be formed. The majority of organic dyes used for bacteria. The Romanowsky stains, such as Giemsa stain, color the organisms more deeply and frequently reveal denser polar staining in the rods. The organisms are motile in young cultures made on suitable media. The use of special flagellar stains, modified Zettnow method¹ or Zettnow-Fontana method, reveals 3 to 5 unipolar flagella (Fig. 44, No. 4). Living organisms are somewhat difficult to see by direct illumination, but darkfield illumination is an excellent means of studying their morphology and motility. Non-motile spirals, which are considered as abnormal cast-off flagella, may be seen with darkfield illumination in cultures.

The bartonella parasitize the erythrocytes during acute Oroya fever and cause the rapid destruction of the cells, reducing the total count of erythrocytes in some cases to less than one million per cmm. The position, numbers and shape of the organisms on the red blood cells are shown in Figure 44, No. 1. The organisms are also taken up by the proliferating endothelial cells in the liver, spleen, lymphatic glands and intestines, and grow as compact masses (Fig. 44, No. 2). The parasites are also to be found in the proliferating endothelial cells of the developing verrugas in the second phase of the disease.

III. **Diagnosis.**—Individuals coming down with a prostrating febrile illness who live or who have visited and slept in areas endemic for Carrion's disease, should be examined for infection with *B. bacilliformis*. There are cases recorded in Peru where individuals slept but a single night in the endemic zone and contracted the disease. Bloodsucking and night-flying insects or sandflies of the genus *Phlebotomus* are incriminated as the vectors of the disease. (See Fig. 92, page 708.)

The diagnosis of the infection depends on the demonstration of the etiological agent, *Bartonella bacilliformis* by (a) direct examination of the blood and tissues, and (b) cultivation of the blood or tissues. A recently



4, flagella of *B. bacilliformis* (silver preparation). (Photograph 2, made from slide in collection of Dr. D. Weinman)

Thin blood films (free from debris and dust particles) should be made and stained with Giemsa stain. If the parasites are present, careful microscopic examination will reveal the typical organisms in their characteristic position on the red cells. Stained impression films made from pieces of biopsied verrugas may also reveal bartonella within endothelial cells.

Isolation of Organisms.—Since the organisms are not always detectable in stained blood or impression films, suitable media should be inoculated with several drops of sterile blood or verrucous tissue and incubated at 28° C. in an effort to grow out the organisms. Noguchi's leptospira semi-solid medium was originally used for this purpose. Growth becomes grossly visible near the surface of the medium in about five to ten days. Blood agar has also given some success. More recently, however, a variety of liquid, semi-solid and solid media^{2,4,8}, cell-free tissue cultures,⁷ and the developing chick embryo⁵ have been used with better success. The formulæ of only those media which have been highly successful in our hands are given. The Geiman semisolid and solid media are satisfactory for primary isolation of *B. bacilliformis*, for routine cultivation and preparation of uniform suspensions for agglutination reactions and vaccines. Growth on these media becomes apparent in forty-eight hours at 28° C. and fine suspensions of organisms equivalent in density to the No. 1 MacFarlane barium sulfate standard can be prepared from the five to seven-day growth on two slants of the solid medium suspended in 1 cc. of physiological saline solution. The liquid medium gives excellent growth also, but the yield of organisms from an equivalent volume of the solid medium is greater.

(a). *Geiman Semi-solid Medium.*²—Base agar—2 gm. Bacto-agar, 8.5 gm. sodium chloride, 1000 cc. of distilled water. Dissolve ingredients and adjust to pH 7.6 to 7.8. To 100 cc. of this base medium, dissolved and cooled to 45° C., add 10 cc. of sterile 5 per cent aqueous Bacto-tryptone adjusted to pH 7.6 to 7.8, 10 cc. of fresh sterile rabbit or sheep serum, and 0.2 cc. ascorbic acid-glutathione solution (10 mg. ascorbic acid and 40 mg. glutathione in 20 cc. Ringer's solution filtered with a Berkefeld N filter). Dispense in sterile test tubes, incubate to test sterility, insert rubber stoppers to prevent evaporation if desired, and inoculate. At 28° C. growth becomes grossly visible at thirty-six to forty-eight hours and very heavy about the fifth day. Minute inocula that fail to grow out on leptospira medium develop very readily on this medium. For that reason the medium is very useful for isolation of the human bartonella and for routine maintenance of cultures. The medium is colorless and transparent, characteristics which facilitate early detection and description of growth. If tubes are kept at room temperature, subcultures can be made with safety at intervals of six weeks to two months.

(b). *Geiman Solid Medium.*—Base agar—20 gm. Bacto-agar, 20 gm. Bactotryptone or Bacto-proteose peptone No. 3, 5 gm. sodium chloride dissolved in 1000 cc. distilled water and adjusted to pH 7.6 to 7.8. To 75 cc. of sterile and dissolved base agar cooled to 45° C. add 25 cc. of fresh rabbit or sheep serum or for economical reasons, 25 cc. of whole defibrinated blood and 0.2 cc. ascorbic acid-glutathione solution. Place sufficient quantities in test tubes to make a short butt and a long slant. When slants are set, place sterile rubber stoppers in the tubes to maintain a moist slant and incubate for sterility. Store in refrigerator until used.

Inoculate the slants with *B. bacilliformis* by the loop method or with a pipet and incubate at 28° C. Growth follows the inoculated streak, becoming grossly visible within twenty-four hours, reaching a maximum in seven to fourteen days also in the water of condensation.

The colonies are mucoid and slightly opaque reaching a maximum diameter of 1 to 1.5 mm. Heavy growth on the slant is confluent, having a characteristic mucoid and opaque appearance. If the growth is harvested in five to seven days in physiological salt solution, measurable quantities of bartonella can be obtained and a fine suspension of organisms can be prepared for immunological studies and making of a vaccine. Formalized (0.4 per cent) suspensions are used for the agglutination test and vaccine.

(c). *Other Culture Methods.*—Another medium which appears to be satisfactory for the growth of *B. bacilliformis* is the blood-glucose-cystine agar devised by Francis for the growth of *P. tularensis*.¹ Jimenez and Buddingh⁵ readily cultivated the organisms at 25° to 28° C. in the allantoic fluid and the yolk sac of the developing chick embryos after inoculation of eight to twelve-day embryos. This author has confirmed the work, but found incubation at 32° C. preferable for the survival of the embryos. The use of the developing chick embryo has no apparent advantage over the use of cell-free media.

IV. *Relationship to Other Organisms.*—Throughout the above discussion of *B. bacilliformis*, morphological and cultural similarities to those of the *Rickettsiæ* are apparent. Nevertheless, the differences are considered sufficient to justify keeping the parasites in different genera.⁶ In the first place the *Rickettsiæ* are obligate intracellular parasites and they require the presence of living or surviving cells for growth in culture media. Secondly, the *Rickettsiæ* do not produce a severe anemia or parasitize the erythrocytes. Thus, the general similarities of *B. bacilliformis* to *Rickettsiæ* are overbalanced by physiological considerations which indicate basic differences in growth requirements and pathogenicity.

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PART V

Rickettsiæ and Filtrable Viruses

CHAPTER XXXII

THE RICKETTSIÆ

By HARRY PLOTZ

GENERAL CHARACTERISTICS

THE rickettsiæ form a group of microorganisms which inhabit the tissues of insects (arthropods) and which are intracellular in habitat; in other words rickettsiæ
produced recog-
nized They

are: (1) the typhus fever group; (2) Rocky Mountain spotted fever group; (3) tsutsugamushi fever group; and (4) "Q" fever. While trench fever is probably a rickettsial disease it has not been transferred to animals and adequate studies have not been made to establish its relationship. Psittacosis, trachoma and lymphogranuloma venereum are generally regarded as not belonging to the rickettsial group.

Rickettsial diseases of man may be defined as self-limited specific infectious diseases, transmitted by arthropods and characterized by sudden onset, with headache, fever and usually a rash. "Q" fever is the exception as a rash has not been described for this disease. The chief pathological lesions occur in the blood vessels.

The rickettsiæ are small pleomorphic microorganisms, usually seen as short bacillary or diplobacillary forms. The smaller forms are about $0.3\ \mu$ in length, but larger forms may be seen. When growth is established in yolk sacs of developing chick embryos the rickettsiæ are larger than in animal tissues. In heavily infected cells, the organisms appear as minute coccoid bodies usually in diploid formation. Rickettsiæ are usually seen in cells. In smears, extracellular forms may be found especially in preparations from tissue cultures but these rickettsiæ come from ruptured cells. Rickettsiæ develop in the cytoplasm of the cell, the only exception being the rickettsiæ of Rocky Mountain spotted fever which are frequently found in the nucleus. Studies of the development of spotted fever rickettsiæ on agar tissue cultures lead us to believe that these rickettsiæ are essentially intranuclear organisms and that they appear in the cytoplasm after intranuclear development. Rickettsiæ stain poorly with most aniline dyes.

color, or by the Machiavello stain with which they stain red and stand out sharply against the blue background of the cell.

The only rickettsiæ which are filterable are those of "Q" fever which will pass through Berkefeld N and W filters. Rickettsiæ have been grown only in living cells. In this respect they resemble viruses, but since they can be seen readily, they also resemble bacteria, and probably occupy a position intermediate between viruses and bacteria. Rickettsiæ are very unstable agents in that they are easily killed. They can be preserved by keeping them at -70°C . or by freezing and drying.

Human diseases caused by rickettsiæ have a world wide distribution. Wherever man, rodents and ectoparasites common to both exist, rickettsial diseases may occur. Several names have been given the same disease depending upon the different parts of the world where the disease was found. Recent studies have demonstrated the immunological relationship between certain of these disease entities.

RICKETTSIAL DISEASES (IMMUNOLOGICAL RELATIONSHIPS)

<i>Typhus Group</i>	<i>Rocky Mountain Spotted Fever Group</i>	<i>Tsutsugamushi Group</i>
Epidemic or European typhus	Rocky Mountain spotted fever	Tsutsugamushi disease
Endemic or murine typhus	Brazilian spotted fever (Sao Paulo typhus)	Rural typhus of Malaya
Brill's disease	Tobia fever of Colombia	
Tabardillo of Mexico	Fiebre boutonneuse	Mite fever of Sumatra
Fiebre nautique Toulon	Kenja typhus	Tropical or scrub typhus
Manchurian typhus	South African tick bite fever	Queensland coastal fever
Urban typhus of Malaya		
	"Q" Fever Group	
	Australian "Q" fever	
	American "Q" fever	

DISEASES IN MAN CAUSED BY RICKETTSIÆ

<i>Disease</i>	<i>Name of etiologic agent</i>	<i>Usual arthropod vector</i>
I Epidemic typhus	<i>Rickettsia prowazekii</i>	<i>P. humanus corporis</i>
II Murine typhus	<i>Rickettsia mooseri</i>	<i>P. humanus (capitis)</i>
III Rocky Mountain spotted fever	<i>R. (Dermacentorzenus) rickettsii</i>	<i>Xenopsylla cheopis</i>
Brazilian spotted fever (Sao Paulo typhus)	<i>R. (Dermacentorzenus) rickettsii</i>	<i>Ceratophyllus fasciatus</i>
Colombian spotted fever	<i>R. (Dermacentorzenus) rickettsii</i>	<i>Dermacentor andersoni</i>
Fiebre boutonneuse, Kenja typhus	subsp. <i>conora</i>	<i>Dermacentor variabilis</i>
South African tick bite fever	subsp. <i>puperi</i>	<i>Amblyomma americanum</i>
IV Tsutsugamushi fever (Scrub typhus)	<i>Rickettsia orientalis</i>	<i>Amblyomma cajennense</i>
V "Q" fever—Australian	<i>Rickettsia burneti</i>	<i>Rhipicephalus sanguineus</i>
American	subsp. <i>diaporica</i>	<i>Hemaphysalis leachi</i>
		<i>Amblyomma hebraeum</i>
		<i>Trombicula akamushi</i>
		<i>Trombicula deliensis</i>
		<i>Hemaphysalis humerosa</i>
		<i>Dermacentor andersoni</i>
		<i>Amblyomma americanum</i>

be employed in distinguishing rickettsiæ by (1) behavior in experimental animals; (2) rickettsial cultures; (3) Weil-Felix agglutination, and (5) rickettsial complement fixation and agglutination.

Isolation of the Infectious Agent.—The guinea pig is most frequently employed for isolating the agent. Healthy male guinea pigs, weighing

100 to 500 gm. are used. Preferably, the temperature of the animal is taken and recorded for about one week prior to inoculation in order to insure obtaining healthy animals. The maximum normal temperature of the guinea pig may be regarded as 39.9°C . (103.8°F .); any temperature above this is considered as fever under usual laboratory conditions. Temperatures are usually taken in the morning before the animals are fed. The guinea pig is held by supporting the lower back of the animal in the palm of the left hand, holding the thumb on the lower abdomen and spreading the legs apart. A clinical thermometer with rounded bulb coated with vaseline is gently inserted into the rectum, care being exercised not to perforate the intestine. The thermometer is inserted for a distance of about 2 inches, and maintained for one minute. If the thermometer is not obtained. With a little care and easily made. The temperature

the patient in all of the rickettsial diseases in the same manner. Successful isolations are more frequent when blood is obtained early in the disease. Ten cc. of blood are removed from the arm vein and 5 cc. are inoculated intraperitoneally into two guinea pigs. If more than 5 cc. of blood are inoculated intraperitoneally into a guinea pig the animal may die. It is best to inoculate the blood immediately upon its removal from the patient.

When blood is obtained late in the disease, it is best to permit the blood with sterile in Ringer's and these antibodies may mask the effect of the virus when whole blood is inoculated into animals, hence, it is advisable to remove the serum. After inoculation, the guinea pigs should be placed in a clean cage, being careful not to overcrowd them. Temperatures should be taken each day and recorded on a temperature chart.

TYPHUS FEVER

There are two varieties of typhus fever. Epidemic typhus is the severe louse-borne disease of high mortality. This disease is carried from man to man by the body louse, *Pediculus humanus corporis* and *P. humanus (capitis)*, the disease being transmitted to man by infected louse excreta.

of low case fatality. The reservoir of the virus is the rat, infection being carried from rat to rat by the rat flea—*Xenopsylla cheopis*, or the rat louse—*Polyplox spinulosus*—and from rat to man by the rat flea. Unlike what occurs with lice infected with epidemic typhus, the rat flea and rat louse do not die of the murine typhus infection. Murine typhus has been found in all parts of the world where the epidemic disease exists, but it may exist alone as in the Southern U. S. While endemic typhus is commonly carried to man by the rat flea, it is possible that it may be transferred by the human louse. The conversion of one strain to another has not been accomplished experimentally.

In epidemic typhus the period of incubation in the guinea pig is about

eight to twelve days. During this period the temperature remains about 38.5°C . to 39.6°C . At the end of the incubation period there is a rise of temperature about the ninth day, followed by a fall and then a rise to 40.2°C . or 40.8°C . The fever is maintained for about five or six days when

CLINICAL CHART

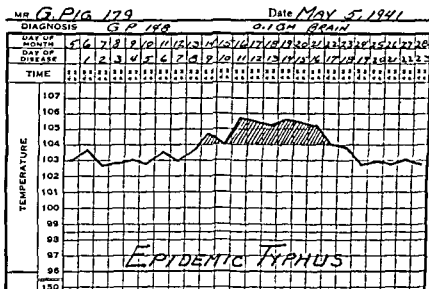


Fig. 45

CLINICAL CHART

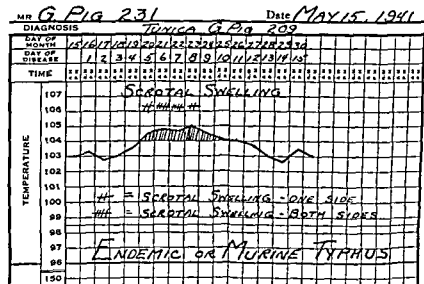


Fig. 46

it drops by lysis. The guinea pig shows no other symptom of disease, and there is no mortality. Occasionally in guinea pigs the infection is latent and is characterized only by the development of immunity.

The infectious agent is passed to other guinea pigs in the following manner. The guinea pig is sacrificed during the febrile period preferably

on second or third febrile day by ether anesthesia. The brain is removed aseptically and thoroughly ground in a mortar with sterile alundum or sterile sand. Ringer's solution or saline is added to make a 10 per cent brain suspension. Since the brain of a 400 to 500-gm. guinea pig weighs about 3 gm., 30 cc. of the diluting fluid are added. One cc. or about 0.1 gm. of brain suspension is inoculated intraperitoneally into each of two or three normal guinea pigs. Cultures should be made to control the sterility of the inoculum. A careful autopsy should be performed to determine whether

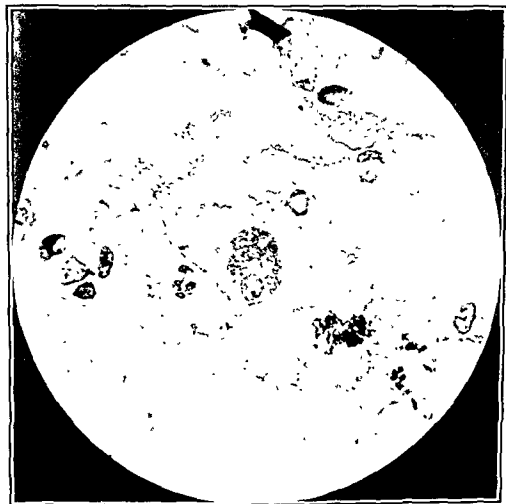


FIG. 47.—Neill-Mooser bodies. Murine typhus fever.

any pathological lesion—such as pneumonia or peritonitis, can be found which might explain the fever. In epidemic typhus the only gross pathological lesion found is a slightly enlarged spleen with slightly prominent Malpighian corpuscles.

In murine typhus, the period of incubation in the guinea pig is usually shorter, varying from four to six days. The temperature then rises to 40.0°C . or 103.5°F ., remains elevated four to six days, and falls by lysis. A characteristic of murine typhus in the guinea pig is scrotal swelling which usually appears on the first or second day of fever. The scrotum

appears prominent, firm, red and swollen and the testicles cannot be forced back through the inguinal ring. When the tunica vaginalis is removed it is found adherent, edematous and may be hemorrhagic in typical infections. Slide preparations are made by scraping the exudate



FIG 48.—Normal guinea pig scrotum.

from the parietal peritoneal surface. After fixing by heat, the preparation is stained with the Machiavello stain. Large serosal cells are found which are literally filled with rickettsiæ which appear in the cytoplasm of the cell and do not invade the nucleus. These cells are called Neill-Mooser bodies and are characteristic of murine typhus in the guinea pig. It takes some practice to find these cells, but a preliminary examination with the



FIG 49 —Scrotal swelling in murine typhus fever.

low power will reveal their presence and examination with the oil immersion lens will identify them. Scrotal swelling does not always occur in small guinea pigs and it is for that reason that guinea pigs weighing 400 to 500 gm. are always used. The guinea pig does not die from the infection.

TYPHUS FEVER

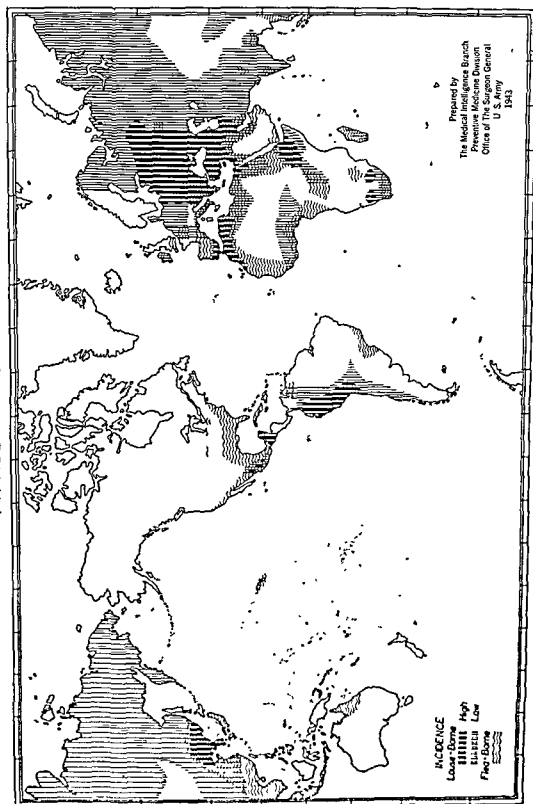
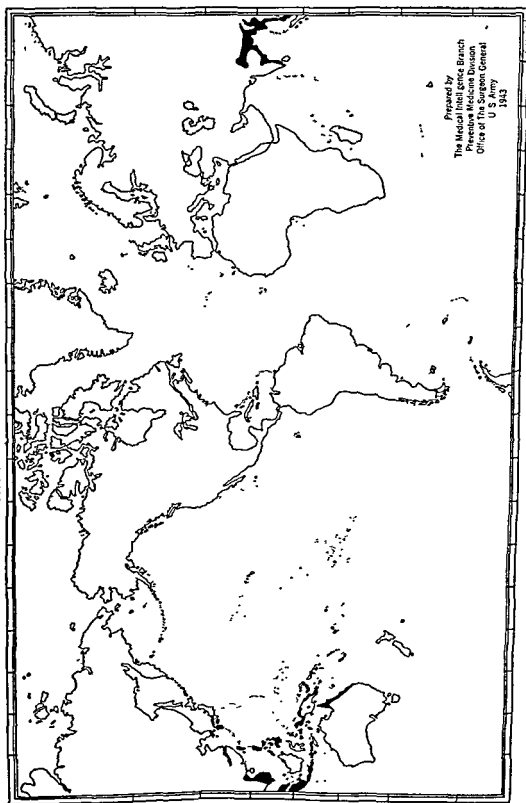


FIG. 60.—Geographical distribution of typhus fever.

MITE TYPHUS



Prepared by
The Medical Intelligence Branch
Preventive Medicine Division
Office of The Surgeon General
U. S. Army
1943

FIG. 51.—Geographical distribution of mite typhus.

The infection is passed from guinea pig to guinea pig as follows: Sacrifice the animal by ether anesthesia on the first or second day of scrotal swelling; shave the abdomen and upper thighs and make a "V" shaped incision from the middle of the abdomen down to both groins and dissect back the skin. Liberate the tunica by blunt dissection and remove both the tunica and testicles. Turn the tunica inside out so that the exudate which appears on the inner surface can be washed off. Place tissue in a 150-cc. Erlenmeyer flask containing glass beads and add 20 cc. of Ringer's solution or saline. Shake tissue actively for a few minutes to liberate the exudate on the surface of the tunica and testicles, and inoculate 1 cc. amounts of this material intraperitoneally into other guinea pigs.

While scrotal swelling is the usual characteristic in murine typhus in the guinea pig and not in epidemic typhus, this rule is not absolute. There are some strains of epidemic typhus which occasionally produce scrotal swelling in the guinea pig, but subsequent passages will induce a disease without scrotal swelling. When scrotal swelling appears in epidemic typhus, examination of the tunica vaginalis may reveal an occasional cell containing a few rickettsiae but typical Neill-Mooser bodies are never seen. In addition to the differences noted in the guinea pig, epidemic typhus may be distinguished from murine typhus by the reaction following inoculation in rats. When endemic typhus is inoculated intraperitoneally into rats a febrile illness is produced, while with the epidemic strain an inapparent infection occurs. In male rats the endemic disease produces scrotal swelling with many serosal cells of the tunica vaginalis filled with rickettsiae, while this does not occur with the epidemic strain. The endemic strain can be easily passed from rat to rat by inoculating a brain suspension intraperitoneally. The virus of endemic typhus remains latent in the brain of an infected rat for months after the initial febrile reaction. In epidemic typhus the virus soon disappears and infection cannot be carried in successive passages.

Immunity tests in guinea pigs may be performed fourteen days or longer after the temperature has returned to normal. One attack of epidemic or endemic typhus in the guinea pig confers a complete immunity to a subsequent inoculation of epidemic or endemic typhus.

ROCKY MOUNTAIN SPOTTED FEVER

This disease is widespread throughout the United States even though not more than a thousand cases occur in any one year. In the west the vector is the wood tick, *Dermacentor andersoni*, while in the east the common vector is the dog tick, *D. variabilis*. *Amblyomma americanum* has recently been found infected in nature. Ticks are the only vectors known to transmit this disease, the virus appearing in the ticks' tissues, ova and feces, and being transmitted hereditarily. While spotted fever may have a high case fatality rate in certain areas, the average fatality rate for the different regions of the country as a whole is about the same.

While *D. andersoni*, *D. variabilis* and *A. americanum* are the known natural vectors, the rabbit tick, *Hæmaphysalis leporis-palustris*, is probably an important factor in maintaining the infection alive in nature. Of the four potential or experimental carriers, two are parasites of man, *A. cajennense*, and *D. occidentalis*. The other two that may be of lesser importance

in nature in this country are *D. parumapterus*, a rabbit parasite, and *Rhipicephalis sanguineus*, the brown dog tick.

Brazilian spotted fever (Sao Paulo typhus), exanthematic typhus of Minas Geraes, and Tobia fever of Colombia are forms of spotted fever which are immunologically identical with the American disease. The probable carrier is *A. cajennense* at least in Brazil.

related to spotted fever, but a spotted fever vaccine does not immunize against it. The rickettsiæ of *fièvre boutonneuse* produce a scrotal swelling in guinea pigs and the virus may be passed by inoculation of tunica washings. Kenya typhus is related to *boutonneuse* fever and is transmitted by the same species of tick.

CLINICAL CHART

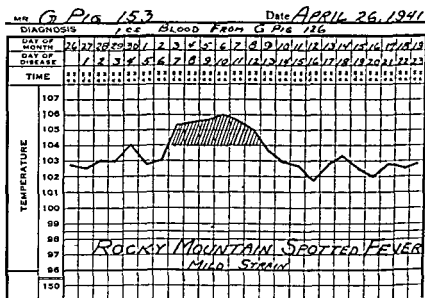


FIG 52

One of the South African tick bite fevers is related to *boutonneuse* fever. It occurs in South Africa from southern Rhodesia to the Cape. Natural infection has been found in *Hæmaphysalis leachi* and *A. hebraeum*.

Since the chief vectors in spotted fever are so-called three-host-ticks, *i. e.*, they seek a new host in each feeding stage; the immature stages feeding on rodents and small animals, the adults on large, wild and domestic animals, and accidentally on man, the opportunities for transferring infection are great. The infectious agent has not been isolated from rodents captured in nature. Native ground squirrels, field mice and related rodents are probably the important susceptible animals.

The infectious agent is isolated from the blood of the patient in exactly the same manner as has been described for epidemic typhus. There is a great difference in virulence of strains for animals and this virulence bears little relationship to the severity of the disease in man. A strain from a fatal case in man may cause only a mild reaction in guinea pigs, while a

strain from a mild case in man may give rise to a severe fatal infection in guinea pigs. Strains isolated from cases of spotted fever occurring in the Bitter-root Valley usually produce severe reactions.

The mild infection in the guinea pig is characterized by an incubation period of about six days, followed by a febrile period of four to five days.

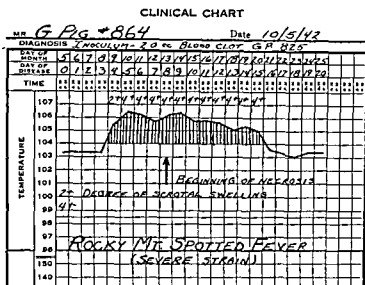


FIG. 53'



FIG. 54.—Scrotal necrosis in Rocky Mountain spotted fever—severe strain.

There is no scrotal swelling and the guinea pig does not die of the infection. With severe infections, the period of incubation may be two to five days followed by a high temperature, scrotal swelling and death of 98 per cent of the animals. The scrotal swelling in Rocky Mountain spotted fever is different from that which occurs in murine typhus. The scrotum is swollen, deep red, and becomes dark purplish-black or even

gangrenous with necrosis followed by scar formation if the animal survives. The exudate in the scrotal sac is not as intense as it is in endemic typhus and rickettsiæ are scarce in smears from the tunica surface. Rickettsiæ occur in large numbers however, in the endothelium and smooth muscle tissue of arterioles beneath the surface, both in the scrotum and the testes. Intranuclear forms may be seen.

The infection is passed by removing blood by cardiac puncture during the febrile period, preferably on the second to fourth days and inoculating 1 to 2 cc. intraperitoneally into normal guinea pigs. A guinea pig that has recovered from Rocky Mountain spotted fever is immune to a subsequent inoculation of spotted fever but is not immune to typhus fever.

TSUTSUGAMUSHI DISEASE

Tsutsugamushi disease is distributed along the course of large rivers in Japan. It occurs in Formosa, Sumatra, Malaya, the Philippines and Queensland. In Japan the disease is transmitted by the bite of infected larvæ of a mite, *Trombicula akamushi*. In Formosa and Sumatra it is transmitted by another closely related species of mite, *T. diliensis*. The animal reservoir in Japan is the vole. The common house rat harbors the mite in Formosa while in Sumatra the animal hosts are both the house and field rat. Some of the "Indian tick bite fevers" thought to be mite-borne may be related.

The virus has a low virulence for guinea pigs and has only been transmitted to this animal when the resistance has been lowered as a result of a vitamin deficient diet. The disease has been transmitted to rabbits by intratesticular inoculation. Intraocular injection in the rabbit results in an ophthalmitis with the presence of rickettsiæ in the endothelial cells overlying Descemet's membrane. This disease confers an immunity to itself but not to typhus or Rocky Mountain spotted fever.

"Q" FEVER

The virus of "Q" fever was first identified from cases occurring in Queensland, Australia. In the United States the virus has been isolated from infected ticks. It has been demonstrated in *D. andersoni* collected in Montana and Wyoming, from *D. occidentalis* from Oregon and California. One case of "Q" fever was reported from Montana. The diseases are more defined.

The virus can be isolated from the blood, especially when obtained early in the disease. It can often be isolated from the urine as well. In the guinea pig a period of incubation of from four to eight days is followed by

and engorged or may be spontaneously ruptured.

Guinea pigs injected subcutaneously or intradermally show a marked inflammatory thickening of the skin at the site of inoculation, with rickettsiæ present in the exudate. Transfers are made by passing blood or

material from the spleen on the second or third febrile day. The urine of guinea pigs is infectious.

Inoculated mice develop small necrotic foci in the liver and rickettsiæ are found in the Kupffer cells. The disease confers an immunity to itself but to none of the other rickettsial diseases.

TRENCH FEVER

This disease has not been transmitted to an experimental animal. It has been transferred to human volunteers by rubbing the feces of infected lice into the scarified skin and also by allowing infected lice to feed on human volunteers.

Extracellular rickettsiæ have been described as occurring in the intestines of infected lice, but it has not been proved that these rickettsiæ are the actual cause of the disease since non-pathogenic rickettsiæ have also been described in these insects. Trench fever disappeared after the first World War, but reports indicate that it has again made its appearance in North Africa.

ISOLATION OF RICKETTSIÆ FROM ARTHROPOD VECTORS

The agents causing the above rickettsial diseases may be isolated from the various arthropod vectors which carry them. Infected ticks, fleas, lice and mites are first washed in a weak solution of bichloride of mercury or merthiolate to reduce surface contamination. Ticks must be allowed to feed before attempts to recover the virus are made. After washing in saline, the insects are ground in a mortar, suspended in saline and inoculated intraperitoneally into guinea pigs which are observed for signs of infection as described above.

TABLE 78.—IMMUNITY IN GUINEA PIGS CONFERRED BY VARIOUS RICKETTSIAL DISEASES

	Epidemic typhus	Murine typhus	Brill's disease	Rocky Mt. spotted fever	Tsutsugamushi	"Q" fever
Epidemic typhus	+	+	+	—	—	—
Murine typhus	+	+	+	—	—	—
Brill's disease	+	+	+	—	?	?
Rocky Mt. spotted fever	—	—	—	+	—	—
Tsutsugamushi disease	—	—	?	—	+	?
"Q" fever	—	—	?	—	?	+

PATHOLOGY

The characteristic lesions in spotted fever and typhus occur in the blood vessels of the periphery of the body and of the central nervous system. However, the vascular lesions are more severe in the former disease, in that necrosis of the vessel walls may occur. In both diseases thrombosis is common. In spotted fever, rickettsiæ invade the smooth muscle cells of the media of blood vessels, while in typhus the localization of the rickettsiæ is restricted to the endothelium. Focal lesions occur in the brain of man and of laboratory animals in both diseases. These consist of perivascular collections of cells and areas of glial proliferation. In spotted fever, the brain lesions are frequently accompanied by thrombosis of small cerebral arterioles and by necrosis, while this is not the case in typhus.

The lesions of Brazilian spotted fever correspond in man and in guinea pigs to Rocky Mountain spotted fever. *Fievre boutonneuse* in man differs

from Rocky Mountain spotted fever by the occurrence of a local granulomatous lesion at the site of the inoculation, which usually ulcerates and is accompanied by adenitis of the regional lymph nodes. In guinea pigs the pathology is comparable to Rocky Mountain spotted fever in that animal.

In tsutsugamushi fever the primary pathological changes are a vasculitis and perivasculitis with slight tendency toward thrombus formation. The perivascular extension of the infection involves the parenchyma of various organs. Lesions occur in the heart, lungs and brain.

The lesions of "Q" fever in the guinea pig consist of perivascular accumulations of lymphocytes and rare focal brain lesions like those seen in typhus and spotted fever. Granulomatous lesions, with giant cells, are also described in the spleen, liver and other organs.



FIG. 55.—*Rickettsia prowazekii*. Yolk sac culture.

The rickettsiae in sections are stained by the Machiavello stain (Pinkerton).

1. Fix tissue in Regaud's fluid, cut thin paraffin sections and run through xylene and graded alcohols to distilled water in the usual way.
2. One per cent aqueous methylene blue overnight.
3. Decolorize in 95 per cent alcohol.
4. Counterstain in 0.25 per cent aqueous basic fuchsin for thirty minutes.
5. Decolorize rapidly (about three seconds) in 0.5 per cent citric acid.
6. Differentiate rapidly in absolute alcohol.
7. Clear in xylene and mount in gum dammar.

The rickettsiæ stain deep red and are easily distinguishable, even though the surrounding tissue is also partly red.

The following method stains the rickettsiæ deep blue against a background which is partly red and partly blue.

- 1 to 5. Procedures as above.
6. Wash lightly in distilled water.
7. Counterstain again in 1 per cent aqueous methylene blue for five seconds.
8. Differentiate in 95 per cent alcohol.
9. Absolute alcohol and xylene. Mount in gum dammar.

RICKETTSIAL CULTURES

Rickettsiæ can be grown by various methods provided living cells are used. The methods include the classical plasma clot, the suspended cell medium, the agar tissue culture method and the yolk sac of developing

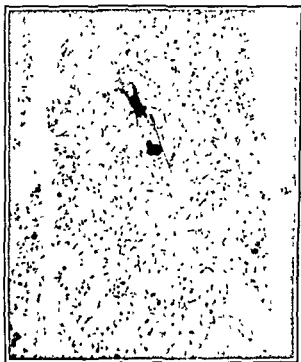


FIG. 56.—*Rickettsia moovers*. Yolk sac culture.

aid that in *in vitro* cultures
ls while viruses grow better

Yolk Sac Method.—Yolk sac cultures are started and maintained as follows. The brain or spleen of an infected guinea pig is removed aseptically on the second or third febrile day of the disease and ground up in a mortar with alundum. A 10 per cent suspension is made in Ringer's solution and gently centrifugalized to bring down any large particles of tissue. The suspension is then inoculated into six-day fertile chick eggs. The eggs should be candled before use so as to be certain that the embryos are alive. The broad end of the egg is sterilized by means of a 3 per cent iodine solution and a small hole is made with a dental pick or drill.

A 21-gauge needle $1\frac{1}{4}$ inches long is plunged perpendicularly through the egg. Then 0.5 cc. of infectious material is injected. The hole is sealed with vasel-
 The hole is sealed with
 (part beeswax, 1 part castor oil).
 The eggs are returned to the incubator which is maintained at 35° C. and are turned daily to prevent the embryo from becoming attached. Each day the eggs are candled to determine whether the embryo is alive. After from four to six days the embryo is found sluggish in its movements or dead. The egg should be opened just as soon as the embryo dies, for if incubated thereafter, the rickettsiæ decrease in numbers and finally disappear. The yolk sac is removed aseptically from the wide end of the egg, by removing the shell over the air sac and either extracting the yolk sac by means of forceps or by inverting the entire egg contents into a Petri dish. A small piece of yolk sac is removed and gently rubbed over the surface of a dry Petri dish in order to remove the excessive amount of adherent yolk material. A smear is then made by rubbing the tissue on a slide and after drying in air, it is fixed by heat and stained by the Machiavello method.

The Machiavello stain is made as follows:

Basic fuchsin	1 gm. in 400 cc. double distilled water
Citric acid	1 gm. in 200 cc. double distilled water
Methylene blue	1 gm. in 100 cc. double distilled water

The basic fuchsin is filtered through filter paper and dropped onto the slide and allowed to remain for four minutes. The fuchsin is washed off with tap water and the citric acid solution is poured on and off quickly, and rinsed off with tap water. Long exposure to citric acid will decolorize the rickettsiæ and they will subsequently stain blue. Methylene blue remains in contact for twenty seconds and the slide is washed in tap water. With this stain the rickettsiæ stain red and the cells blue.

In the first egg passage few or no rickettsiæ may be seen, in which case it is necessary to pass the yolk sac material to other eggs. The yolk sac is ground up in a mortar with alundum and suspended in 20 cc. of Ringer's solution or saline. A series of fertile eggs are again inoculated as above with 0.5 cc. of infectious material. After a few passages a strain will be established and rickettsiæ will be seen in increasing numbers, in successive transfers. The rickettsiæ may appear small in early cultures but in later egg passages they become larger. The rickettsiæ obtained in egg cultures maintain their virulence for guinea pigs.

Agar Tissue Method.—The semi-solid agar tissue medium employed for culturing rickettsiæ is made as follows:

A. Double Tyrode solution	1200 cc. (see below)
Calf or beef serum (cell-free)	800 cc.
Phenol red indicator	64 cc. (see below)
Filter through Seitz pad. Determine sterility. Store in ice-box.	

B. Granulated agar	45 gm.
Triple distilled water	1200 cc.

Autoclave for $1\frac{1}{2}$ hours at 15 pounds. Cool. Place in water bath at 45° C with flask A. When temperature of each flask has reached 45° C, mix, and pour into Kolle flasks made with a special neck holding a No. 6 rubber stopper. Large test tubes may also be used.

Formula for double Tyrode:

Sodium chloride	160 gm.
Potassium chloride	4 gm.
Calcium chloride	4 gm.
Magnesium chloride	2 gm.
Sodium acid phosphate	1 gm.
Triple distilled water	1000 ml.
Sodium bicarbonate	20 gm.
Dextrose	20 gm.

Phenol Red Indicator:

Phenol red powder	0.1 gm
N/20 Sodium hydroxide	5.7 cc.

Grind phenol red powder in NaOH, then add triple distilled water up to 250 cc.

Ten-day chick embryo cells are finely cut with scissors, the infectious material is brought in contact with the cells and after thorough mixing is kept in the ice box for one-half hour. The material is then spread on the is tightly Mountain transplants are made by scraping off the cells with 3 cc. of saline and grinding the material with alundum in a pyrex hand grinder. After the alundum has settled the supernatant is removed and brought in contact with fresh chick embryo cells.

WEIL-FELIX AGGLUTINATION

The agglutination of certain strains of *Proteus vulgaris* by sera of patients infected with certain rickettsial diseases has been used as a helpful aid in diagnosis. While the *Proteus* bacillus is in no way related to rickettsiae and hence has no etiological significance, it has been suggested that the agglutination reaction depends upon the possession of a common antigenic factor by the two organisms.

The *Proteus* strains commonly used are the OX₁₉, OXK and OX₂. The high titre Mountain nt in these diseases. Since a OX₁₉ occur in typhus and spotted fever, to differentiate these diseases. OX₁₉ Rocky Mountain spotted fever.

In tsutsugamushi disease agglutinins for the OXK organism occur in relatively high titre while those for OX₁₉ are low or absent. Neither OX₁₉ nor OXK agglutinins occur in "Q" fever. Since the Weil-Felix reaction had not been described when trench fever occurred during the first World War, we do not know what type agglutination occurs in this disease.

TABLE 79.—WEIL-FELIX AGGLUTINATION IN VARIOUS RICKETTSIAL DISEASES

	OX ₁₉	OXK
Epidemic typhus	+	—
Murine typhus	+	—
Brill's disease	+	—
Rocky Mountain spotted fever	+	—
Tsutsugamushi disease	—	+
"Q" fever	—	—
Trench fever	?	?

Only the O or non-motile variant of the *Proteus* bacillus is used for the agglutination reaction since it is this antigen which reacts specifically with sera from rickettsial diseases. The purity of the strain should be controlled frequently by streaking on a veal agar infusion plate. After twenty-four hours incubation, the smooth, non-spreading O type colonies are selected and transferred to tubes of dry agar and broth. If the organism is non-motile the culture may be used for the agglutination test. All cultures should be maintained on dry agar slants. Lyophilization is helpful in main-

agar cultures in 0.85 per cent saline. The turbidity of the suspension is adjusted to that of tube 3 of the McFarland nephelometer scale. The killed antigen is prepared as follows: A smooth non-motile strain is grown on agar in Kolle flasks, and the culture is washed down with sufficient saline to make a heavy suspension, and 0.5 per cent formalin is added. This represents the concentrated stock. This antigen is then diluted with saline when needed to have a turbidity equal to tube 3 of the McFarland scale.

A macroscopic agglutination test is performed by thoroughly mixing 0.5 cc. of serum dilution and 0.5 cc. of antigen suspension. Serum dilutions of 1 to 10 through 1 to 640 (final dilution 1 to 20 through 1 to 1280) are usually sufficient. A control tube containing 0.5 cc. of antigen and 0.5 cc. of saline should be included as well as a positive serum control. Tests and controls are incubated in the water bath at 37° C. for two hours followed by storage overnight in the ice box. Complete agglutination is indicated by absolute clearing of the supernatant fluid and by settling of the organisms in large white particles at the bottom of the tube. Partial agglutination is indicated by incomplete clearing of the supernatant fluid and diminution in size of the bacterial clumps. When the tubes are shaken, granular

in titre during the late convalescence. For this reason an agglutination reaction should be performed as soon as a rickettsial disease is suspected and tests should be repeated at frequent intervals during the course of the disease. Of great diagnostic significance is a rise in agglutination titre. A single positive agglutination of 1 to 80 is of no diagnostic value because non-specific agglutination may occur in other diseases. A single agglutination titre of 1 to 1280 probably means that the patient is suffering from some rickettsial disease. An agglutination titre of 1 to 50,000 has been recorded in some cases of typhus fever. The diagnosis should not be made on a positive Weil-Felix agglutination alone; further clinical or laboratory evidence should be obtained. A consistently negative Weil-Felix may be obtained in typical cases of typhus fever.

Rickettsial Complement Fixation and Agglutination.—The complement-fixation reaction has become a useful diagnostic method in rickettsial diseases. It may be used in epidemic and murine typhus, spotted fever and "Q" fever, and probably for other rickettsial diseases.

Endemic, epidemic and "Q" fever antigens may be prepared from infected yolk sacs of developing chick eggs. In addition, endemic typhus antigens may be prepared from the lungs of mice and rats infected by the

intranasal route; "Q" fever antigens from spleens of infected mice; and spotted fever antigens from yolk sac cultures or better from cultures grown by the agar tissue culture method.

Antigens from yolk sac cultures may be prepared by several methods. The yolk sacs of infected chick eggs are removed as soon as the embryo dies, usually on the fifth or sixth day after inoculation. The yolk sacs are removed and collected in a heavy pyrex bottle carrying glass beads and shaken in the shaking machine for one hour. A 20 per cent suspension by weight is made in phosphate buffered saline pH 7 containing 0.5 per cent of formalin. The suspended material is placed in a bottle containing a syphon system and inverted so that the yolk cream can rise to the top. The bottle is kept in the ice box for forty-eight hours. The lower liquid is withdrawn and centrifugalized in the angle centrifuge for two hours at 5,000 r.p.m. in the cold room. The yolk cream which rises is carefully removed, along with the supernatant which is discarded. The sediment which contains the rickettsiae is resuspended in the same phosphate buffer with 0.2 per cent formalin and made up to the original volume. The suspension is purified by centrifugation at 1000 r.p.m. to remove any large particles and at 5000 r.p.m. to bring down the rickettsiae. These rickettsial suspensions are then washed in buffer solution three to five times. The final rickettsial suspension contains 0.2 per cent formalin in buffered saline.

Spotted fever antigens are preferably prepared from agar tissue cultures. Rich cultures are washed down with saline containing 0.5 per cent formalin and the suspension is frozen and thawed five times. The suspension is then centrifugalized at low speed to bring down tissue particles, and then the supernatant is centrifugalized in an angle centrifuge at 5000 r.p.m. for two hours in the cold room. The sediment (rickettsiae) is suspended in saline merthiolate (1 to 10,000).

The test is carried out by using 0.25-cc. amounts of inactivated serum in dilutions ranging from 1 to 3 to 1 to 192 or higher, 0.5-cc. amounts of complement representing two full units, and 0.25-cc. amounts of antigen (2 units) whose titre was determined by preliminary titration. Fixation is carried out for twenty hours in the ice box following which 0.5 cc. of sensitized sheep cells are added and incubated at 37° for one-half hour. Equal amounts of a 3 per cent suspension of sheep r.b.c. and of diluted amboceptor, 3 units in each 0.25-cc. volume, are mixed thirty minutes before the sensitized cells are added to the test.

Specific fixation reactions are obtained in each disease. Typhus fever can easily be distinguished from Rocky Mountain spotted fever. In typhus fever, there may be some cross fixation between epidemic and endemic typhus, but fixation with the homologous rickettsial antigen always occurs in a higher titre. It is thus possible to distinguish epidemic from endemic typhus.

Complement-fixing antibodies in typhus usually appear in the late febrile period and gradually increase in titre. In a series of cases of epidemic typhus fever recently studied, a positive fixation with a rise in titre was obtained in all cases. A positive reaction appears to persist long after convalescence. Complement-fixing antibodies have been demonstrated twenty-six years after the initial attack of epidemic typhus fever. Since

the Weil-Felix agglutination disappears in late convalescence, the complement-fixation test is a better criterion of past infection.

Purified rickettsial suspensions may be used in performing agglutination tests; these tests are best performed by the macroscopic method. Agglutinins appear early and increase in titre during the course of the disease.

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Intracellular affinity is another characteristic of viruses, in that they flourish best within cells. Much remains to be learned of the nature of this apparent obligate relationship between viruses and cells and the effect which one exerts upon another. Necrosis of invaded cells often occurs but it is not clear whether it is the effect of toxic products, or the interference with cell metabolism which results from the multiplication of the virus within the cell. Nevertheless, in the course of this intracellular multiplication, abnormal, intranuclear or intracytoplasmic structures, designated as *inclusion bodies*, may be produced. These attain a size far larger than that of the individual virus "unit," and their detection through special staining methods is, under certain circumstances, of considerable diagnostic aid. In intracytoplasmic location, these bodies probably represent developmental processes of the virus (or aggregations of virus units) within the invaded cell. Tissue from the invaded cell may take part in their structure. In some instances inclusion bodies can be actually freed from the cell structure, as in the case of *elementary bodies* in vaccinia.

Selective localization or special affinities for particular types of tissue within certain animal and plant hosts represent another characteristic common to viruses. For instance, there is a group of viruses which show a predilection for the invasion of nervous tissue, the *neurotropic viruses*—such as rabies virus, poliomyelitis virus, and several of the encephalomyelitis group of viruses. Others have been classed as *dermotropic*—such as smallpox virus or chickenpox; and still others as *riscerotropic*—such as yellow fever virus and mumps.

From this brief summary of properties of viruses we find that it is difficult to "separate" or isolate viruses from the tissues they infect or invade. Clinical methods of demonstrating their presence and of diagnosis, and attempts to understand their properties, are often completely dependent on the inoculation of animals and the study of animal tissues.

A list of the more common viruses known to infect man:

I. *Neurotropic Viruses:*

Lymphocytic choriomeningitis

Rabies

Poliomyelitis

Equine encephalomyelitis (Western, Eastern and Venezuelan)

St. Louis encephalitis

Japanese B encephalitis

Russian spring-summer encephalitis (closely related to Louping ill)

West Nile

Ascending myelitis (Virus B)

II. .

. Vaccinia

Herpes simplex

Foot and mouth disease

Verruca

Molluscum contagiosum

Trachoma

Inclusion blennorrhoea

Epidemic keratoconjunctivitis

III. *Respiratory:*

Common cold
Influenza (Types A, B and X)
Psittacosis (Ornithosis)

IV. *Intermediate:*

Measles
German measles
Lymphopathia venereum
Rift Valley fever
Phlebotomus (sand-fly or Pappataci) fever
Dengue

V. *Viscerotropic:*

Mumps
Yellow fever

DIAGNOSTIC PROCEDURES IN HUMAN VIRUS DISEASES

First steps in the demonstration or "isolation" of active virus consist in the inoculation of susceptible animals, or the inoculation of suitable tissue culture media. These procedures may require several preliminary measures designed to rid the original material of bacteria, which is accomplished either by filtration or by the use of chemical bactericidal (although not virucidal) agents. Indirect methods of "demonstrating" virus include the detection of inclusion bodies through histological methods, or the demonstration of viral *antibodies*.

I. **Collection of Material.**—In the collection of any material for virus work, it is well to remember that with many diseases the virus is more readily demonstrated in the *early days* of the disease than in its later period when antibodies have made their appearance. Blood, sputum or nasal washings, stools, purulent material, and spinal fluid all represent material from clinical cases which may serve as a source of virus. Tissues obtained

best method for preparing the inoculum and the types of animals (or culture media) to be inoculated. In many instances time may necessarily elapse between the collection of the material and the inoculation into a suitable animal or tissue culture, and it is not enough to leave such material at the usual ice box temperature in the hope that the virus may survive for one or two days of such storage prior to inoculation, particularly is this true if material is left in a fluid state.

II. **Preservation of Viruses.**—1. **Freezing.**—A large, wide-mouth thermos jar half filled with pieces of solid carbon dioxide (dry ice) is useful for the temporary preservation of material containing viruses and for the transportation of such material.

For any laboratory which is equipped to work with unstable viruses a low temperature storage cabinet such as the one recently described by Horsfall* is useful. Such a cabinet should be composed of thick-walled, insulated material and can be designed to accommodate about 50 pounds of dry ice (renewed every third or fourth day) together with a number of

* Horsfall, S. L., Jr.: Jour. Bacteriol., 40, 559, 1940.

metal racks (Fig. 57) into which celluloid specimen tubes with metal screw caps* can be fitted (Fig. 58). The use of celluloid instead of glass tubes eliminates the loss of specimens resulting from cracked or broken tubes, and the substitution of metal screw caps for stoppers prevents the unstoppering of tubes due to gas expansion during thawing. The temperature within such a storage cabinet can be maintained at an average of -76°C .

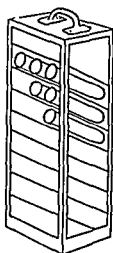


FIG. 57

FIG. 57.—Type of metal rack (with celluloid tubes in place) which is useful in the storage of frozen virus containing material in a solid CO_2 refrigerator. (After Horsfall†)

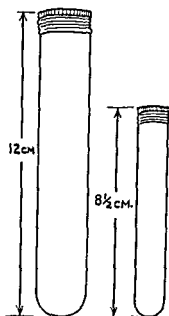


FIG. 58

FIG. 58.—Celluloid tubes with metal screw caps useful for the storage of frozen virus material. (After Horsfall †)

a small bath containing alcohol and dry ice) and rapid dehydration from the frozen state at high vacuum. The process is continuous, conducted in the containers in which the material is to be sealed under the original vacuum.

If the virus to be preserved in this manner is to be dried from fluid material, some viscous substance such as 10 or 20 per cent suspensions of sterile mucin, or 10 or 20 per cent (or undiluted) normal rabbit or normal monkey plasma or serum, should be present as a vehicle in the fluid menstruum in which the virus is to be frozen and dried. If the latter (plasma or serum) is used it is important that it should not contain antibodies which would be effective in neutralizing the particular virus which is to be preserved by this method.

If tissues are to be preserved by this method, they may be ground to a

* Obtainable from the Lusteroid Container Company, Inc., 10 Parker Ave., West, South Orange, N. J.

† Horsfall, S. L. Jr. *Jour. Bacteriol.* 40, 559, 1940

‡ Flosdorf, E. W., and Mudd, S. *Jour. Immunol.* 29, 389, 1935; 34, 469, 1935.

a simple and effective means to render the inoculum bacteria-free. Ether, which is not harmful to some viruses such as poliomyelitis virus and measles virus, is occasionally a useful substance for this purpose. It may be added in amounts of 10 per cent for nasal or oral washings and 15 per cent for stool suspensions. The etherized suspension in a rubber stoppered tube or flask should then be allowed to stand for twenty-four hours in the ice box before inoculating. Ether is, however, harmful to some viruses. Other substances which have been recommended for this purpose include Duponol (sodium lauryl sulfate) which has been recommended in work with poliomyelitis virus, and Zephiran* for work with influenza virus. These bactericidal agents are advised as a substitute for filtration through candles or ultra-filters; for filtration does not always prove satisfactory, inasmuch as most of the virus itself may be retained along with the offending organisms.

IV. Filtration of Viruses.—1. *Types of Filters.*—Earthenware, porcelain and asbestos filters have been largely superseded by collodion membrane filters, although they still have a place in the clinical laboratory. Their limitations result from the fact that the degree of absorption during filtration (through earthenware or porcelain candles) is such that the material to be filtered should contain large amounts of virus. At the same time the concentration of tissue protein should be low, for any large tissue particles in the suspension, are liable to clog the pores of the filter. These particles should be eliminated by preliminary centrifugation of at least 3000 r.p.m. for half an hour.

The following types of filters have been used:

(a) *Berkefeld Filters.*—Made in Germany from diatomaceous earth and supplied in three grades of porosity: V, coarse (pores 8 to 12 μ) which is for clearing solutions and does not retain all bacteria; N, normal (pores 5 to 7 μ) which retains ordinary bacteria as well as some viruses; W, fine (pores 3 to 4 μ) which retains bacteria and some viruses.

(b) *Mandler Filters.*—An American modification of the German Berkefeld, manufactured from kieselguhr, asbestos and plaster-of-Paris which is supplied in three grades: "preliminary," "regular" and "fine," corresponding approximately to V, N and W Berkefeld.

(c) *Chamberland Filters.*—A French type made of unglazed porcelain (kaolin with a little sand). Since the entire candle is made in a single piece, free from joints, it possesses mechanical advantages over the Berkefeld type, and it has the added virtue of being easily cleaned by heating to a high temperature. There are nine grades: L1 (coarse, like Berkefeld V, not retaining bacteria), L1 bis, L2, L3 (like Berkefeld N, pores 2.7 μ , retaining most bacteria), L5, L7, L9, L11 and L13 (finest, retaining some viruses).

(d) *Seitz Filters.*—These are made of asbestos and are quite practical for virus work in the clinical laboratory. Several sizes are made, the smallest of which is the most convenient and consists of an asbestos disc which fits into and is clamped at its circumference in a metal holder. After each experiment the disc is discarded and replaced by a fresh one which can be sterilized in a Petri dish by dry heat. The grades supplied are: K (coarse grade) intended for clarification, EK ("germicidal") which removes ordinary bacteria.

* Science, 56, 543, 1942.

2. **Testing Filters.**—In the case of filter candles, major defects (cracks or leaking joints) can often be detected by immersing the candle in water with the closed end up and attempting to blow air through it. Before using for viruses, all filter candles should be assembled, sterilized in the autoclave, then tested against fluid containing a twenty-four hour broth culture of some bacterial organism such as *B. prodigiosus*. The filtrate should then be cultured and if no growth appears in forty-eight hours the filter may be regarded as tight.

3. **Cleaning Filters.**—This is not easily accomplished. The method recommended by Rivers for filter candles states that if infectious material has been used, the candle should first be immersed in a non-coagulating germicide such as cresol. Débris should then be removed from the outside of the candle with a stiff hand brush with care to avoid rough handling. The candle should then be placed in a metal holder, and water (or salt solution if the fluid filtered contained globulin) should be forced through the filter from within until clean. It may then be boiled for half an hour in 2 per cent sodium carbonate and in several subsequent changes of distilled water. Water should again be forced through the candle until all alkali has been removed. If clogged with organic material Chamberland's candles may be dried in a warm oven and gradually heated in a muffle furnace to a dull red heat and then slowly cooled. Berkefeld filters often crack if so heated.

Sterilization is usually accomplished by autoclaving one hour at 115° C.

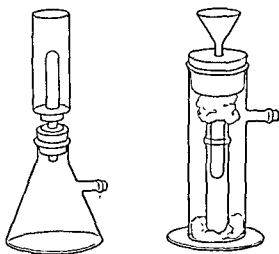


FIG. 59.—Common types of filtration apparatus for: Berkefeld (left) and Chamberland candles (right). The side arm connects with a mercury manometer and a suction apparatus.

4. **Filtration Technic.**—Two types of familiar filtration apparatus are shown (Fig. 59). Both are adapted for the use of negative pressure and the pressure outlet should be connected with a mercury manometer so that its degree can be measured. As a rule pressures should not exceed 35 to 50 mm. Hg.

The fluid to be filtered should be first cleared by centrifugation, or by filtration through paper or some coarse filter. However, many other factors besides that of the turbidity of the original solution affect the process of filtration. Such factors include the amount of positive or negative pressure

applied; the length of time for which it is exerted; the viscosity of the fluid filtered; the pH of the fluid; the number and size of infective elementary bodies in the suspension; the electrical charge of the filter and of the material to be filtered; and the average pore diameter of the filter. If the medium is acid, filters may retain small bacteria and some viruses, which will pass through if it is slightly alkaline. Filtration is facilitated by suspending the material in meat infusion broth or 10 per cent rabbit serum and by first drawing some sterile broth or 10 per cent serum through the filter.

5. **Collodion Membrane Filters (Elford).**—The use of especially prepared collodion membranes has superseded that of earthenware or asbestos filters in many laboratories, an advantage of the membrane being that it does not have many of the undesirable absorptive effects associated with earthenware filters. The task of making suitable collodion membranes for this purpose, however, is difficult and time-consuming. Workers desirous of making their own membranes should consult Elford's original publications and also that of Bauer and Hughes.* Other advantages of these membranes consist in the fact that there is uniformity of porosity which can be secured in a number of different grades.

V. **Animal Inoculation.**—Types of laboratory animals and the routes of inoculation to be used depend upon the type of virus being studied and the character of the inoculum. The usual laboratory animals employed today for clinical work include the following rodents: mice, hamsters, cotton rats, guinea pigs, and rabbits. Other laboratory mammals often used in clinical virus work include ferrets, cats, and monkeys.

1 **Intracerebral Inoculation.**—This is the method of choice for many neurotropic viruses. The inoculum should be relatively free from pathogenic bacteria, for the brain is more susceptible to bacterial infection artificially introduced than are many other parts of the body. Mice should be anesthetized and of six using a total inoculum of 0.03 cc. for each mouse. A

tuberculin syringe may be used with a 27-gauge needle (length $\frac{1}{2}$ inch, or preferably $\frac{1}{4}$ inch). For the intracerebral inoculation of anesthetized guinea pigs and rabbits a heavier needle will be required and the sharp end of a sterile pair of scissors is a useful instrument for drilling a small hole in the skull. The site of inoculation is the parietal lobe back of the eyes and to one side of the midline. An inoculum of 0.1 cc. may be used for guinea pigs and a little larger (0.25 cc.) for rabbits. Amounts much larger than this may, however, cause the acute death of the animal.

For monkeys the size of the intracerebral inoculum depends on the size of the animal; 0.5 cc. is sufficient for a small monkey. A 22-gauge needle, 1 inch in length is suggested and the hole in the skull can be again drilled with the sharp points of a pair of scissors.

All animals inoculated by intracerebral and other routes should of course be examined closely for symptoms each day subsequent to inoculation, and this daily observation should be pursued for an appropriate length of time—three to four weeks is advised for most neurotropic viruses. Rectal temperatures should be taken daily on rabbits, guinea pigs and monkeys and the latter animals should be exercised daily with careful observation for any symptoms. If the inoculated animal dies during

* Bauer, J. H., and Hughes, T. P. J. Gen. Physiol., 18, 143, 1934.

the period of observation, bacterial cultures should be made of the brain and heart's blood in order to eliminate or confirm the presence of an interfering bacterial infection.

2. **Intranasal Inoculation or Instillation.**—It is wise for the laboratory worker doing intranasal instillations to wear a cellophane mask. Mice should be anesthetized and the material may be dropped on the nares with a capillary pipet. With monkeys, anesthetization is not necessary and the material may be dropped from a 5- to 10-cc. pipet, 2 cc. of the

into the nose.

3. **After Subcutaneous and Intraperitoneal Inoculations** (as well as intranasal inoculation) the animals should be observed carefully and if they are large enough, their rectal temperature should be recorded daily. At the end of the period of observation it is generally wise to sacrifice the animal. A careful autopsy should be performed.

VI. **Demonstration of Cellular Inclusions.**—The structure and staining qualities of these bodies, which may be either intracytoplasmic or intranuclear, are variable. They may be large eosinophilic bodies or small basophilic granules. Less common are the small eosinophilic and large basophilic structures. In smears and sections they may be stained with Giemsa stain, eosin-methylene blue, Mann's, or Casteneda's stain.* The demonstration of these bodies may be of considerable diagnostic importance, such as: the cytoplasmic inclusions, known as Negri bodies in rabies, Guarnieri bodies in the epithelial cells of the lesions of smallpox and vaccinia, the psittacosis bodies demonstrable in tissue cultures of psittacosis virus; as well as the acidophilic intranuclear inclusions found in yellow fever, and in lesions induced by herpes virus. The mere finding of an inclusion body in a cell does not necessarily indicate a virus infection; the inclusions must occur constantly in certain infections before their specificity becomes established.

Tissues which are to be sectioned and studied for inclusion bodies should be carefully fixed with special fixing reagents. For this purpose Zenker's fixing reagent containing 5 per cent glacial acetic acid is recommended, as are also Bouin's solution and Sousa's solution.*

VII. **Cultivation.**—Viruses do not multiply on inanimate culture media but propagation may proceed in the presence of living cells of tissue culture. Many variations of technic are used for this purpose, although in general the usual culture consists in preparations of minced embryonic tissue, testis or kidney suspended in serum, plasma tissue extract, or serum

infected fragments of mouse brain, have been largely supplanted in this country by the use of minced tissue in a fluid medium. Only two of these procedures, modified from that of Rivers,† will be described here.

* For the technic of fixing and staining, a Textbook of Histology or a Manual of Staining Methods should be consulted.

† Li, C. P., and Rivers, T. M.: *Jour. Exper. Med.*, 52, 465, 1930.

1. *Technic of Tissue Culture (Modifications of Rivers' Method).**—Tissue is obtained from a nine to twelve-day chicken embryo after the removal of its head and limbs. The embryonic tissue is minced in 3.5 cc. of Tyrode's solution. The Tyrode's solution is freshly prepared every ten days according to the following formula:

NaCl, 8.0 gm.; KCl, 0.2 gm.; CaCl_2 , 0.2 gm.; MgCl_2 , 0.1 gm.; NaH_2PO_4 , 0.05 gm.; NaHCO_3 , 0.5 gm.; glucose, 1.0 gm.; double distilled water q.s. ad. 1000 cc.; pH adjusted to 7.7 and sterilized by filtration through a Seitz filter.

Cultures are prepared by adding 0.1 to 0.2 cc. of the minced chick embryo tissue to each 5 cc. of filtered Tyrode's solution. This amount is placed in a 50-cc. rubber stoppered Erlenmeyer flask. Culture flasks are inoculated with 0.25 cc. of a bacteria-free virus suspension and the cultures are incubated at 37.5° C. for three days. Bacteriological controls should be made.

There are many modifications of the above procedure which should be adapted to the various types of viruses being cultured. These modifications include the addition of an indicator such as phenol red; the addition of whole rabbit serum or serum ultrafiltrate as a growth promoting factor; the incubation of the cultures at room temperature instead of at 37.5° C. For the culture of the common cold virus and influenza virus, Dochez *et al.*† have recommended the following method: Minced ten-day chick embryo is pipeted into culture tubes (2 cm. diameter) in equal amounts; 10 cc. of special peptone broth are then added (sodium chloride-free, made with non-toxic casein peptone, and containing 0.1 per cent gelatin). Sufficient cysteine hydrochloride is then added to give a concentration of 1 to 2000 (cysteine is prepared in 1 per cent solution, neutralized with caustic soda, autoclaved and kept under a vaseline seal). The culture tubes are sealed with vaseline, and stored at 4° C. until required and cultures are initiated by introducing virus-containing material through the vaseline seal.

2. *The Use of the Developing Hen's Egg.*—The use of the developing embryo as a method of propagating viruses has also increased during past years. The method offers a valuable source of virus for a variety of purposes, such as the production of antigen for use in the manufacture of vaccines or of antigens for serological tests.

There are various methods of inoculating the incubating eggs but that advocated by Burnet‡ is given here.

(a) *Source of Eggs.*—A reliable source of fertile eggs is necessary. Eggs should be as fresh as possible, certainly not older than a week when incubation is commenced. They should be kept in a cool place and turned daily until placed in the incubator.

(b) *Preliminary Incubation.*—Any suitable form of commercial egg incubator may be used. In practice the incubator will usually have a number of boxes of eggs at different stages of incubation. A constant

* Cox, H. R., Syverton, J. T., and Olitsky, P. K.: *Proc. Soc. Exper. Biol. and Med.*, 30, 896, 1933.

† Dochez, A. R., Mills, K. T., and Kneeland, Y.: *Jour. Exper. Med.*, 63, 559, 1936.

‡ Burnet, F. M.: *Med. Research Council (Brit.), Special Rep. Series*, No. 220, 1936; Burnet, F. M., and Faris, D. D.: *Jour. Bacteriol.*, 44, 241, 1942.

temperature of 39.5° C. should be maintained. Egg trays should be removed for ten to fifteen minutes daily during which time the eggs are turned. Gloves should be worn when turning eggs.

After about twelve days' incubation the eggs are examined by transillumination. If the embryo is dead, the appearance is usually obvious, but embryos very recently dead may provide difficulty. Points indicating death are: absence of spontaneous movement of the embryo, absence of visible blood vessels, and undue mobility of the embryo on moving the egg. A properly developed and living egg at this stage shows a large dark area representing the embryo and yolk sac. At the blunt end of the egg the air space can be seen, now considerably larger than in the unincubated egg. The limits of this air space are outlined in pencil and on the side of the egg farthest from the line along which the opposite sides of the chorio-allantois have united, an equilateral triangle with sides 1 to 1.2 cm. long is marked.

(c) *Drilling*.—A dental motor fitted with a thin cutting disc is used for this purpose. Care should be taken to avoid the slightest damage to the shell membrane and it is useful to make the cut deeper at the angles than at the middle of the sides of the triangle. The drilling is completed by making a small cut through the compact layer of the shell over the air space. After drilling, the eggs are returned to the incubator. They should be inoculated within two hours. Inflammatory changes develop rapidly in the chorio-allantois beneath the drill marks, and within less than twenty-four hours the membrane becomes adherent to the shell membrane at these points.

(d) *Inoculation*.—The equipment consists of: a small stand of metal or plasticine to support the egg about 3 cm. above the bench level; a straight triangular cutting edge needle mounted in a suitable handle; a medium-sized nail or some similarly pointed object to make the opening into the air sac; capillary pipets, and rubber bulbs.

An opening is first made into the air sac with the nail point. With the needle each corner of the triangle is gently elevated until it is clear that the triangle of shell will lift off easily. If this precaution is not taken it is easy to lift one side of the triangle and force the sharp angle opposite through the shell membrane. In the middle of the area of shell membrane so exposed a drop of sterile fluid (normal saline with 0.04 per cent CaCl_2) is placed. Through this drop a slit is made in the fibers of the shell membrane and enlarged sufficiently to be sure that the saline has come in contact with the upper surface of the chorio-allantois. The details of making the slit are as follows: the needle point should be only moderately sharp and should contact the shell membrane at an angle of about 45 degrees. It is pressed slightly downward until the point engages the fibers of the shell membrane and then raised so as to slit the membrane along the direction of the fibers. The edge of the slit should be raised sufficiently to extend the opening about 3 mm. and to allow a glimpse of the chorio-allantois. Hemorrhage should be completely absent. The egg is now put aside for a minute or two while others are similarly prepared. During this period the saline begins the work of gently separating the chorio-allantois from the shell membrane. The next stage is to complete the formation of the artificial air space by suction with a rubber bulb over the opening into the natural air sac. This should be done very gently and the

- (d) The 50 per cent end points (LD_{50}) are to be calculated according to the method of Reed and Muench,* the titre being expressed as the logarithm of the dilution (to one decimal) (see note in example).
- (e) The neutralization index of a serum can be expressed (not logarithmically) as the ratio of the control LD_{50} titre over the LD_{50} titre of the serum. (\log of LD_{50} of control - \log of LD_{50} titre of serum = X; antilog of X = neutralization index.)

On this basis the following criteria are adopted:

Neutralization index 1 to 9 = negative.

Neutralization index 10 to 49 = equivocal.

Neutralization index 50 or more = positive.

When an equivocal result is obtained the serum should be retested. When in the second test the neutralization index is 1 to 9, the serum should be considered negative; 10 to 49 shall leave the serum in the equivocal category, the final decision being postponed until another test is done on the same serum or on another specimen from the same individual; an index of 50 or more in the second test will put the serum in the positive category.

2. Details of the Neutralization Test for Neurotropic Viruses.—(a) *Virus*.—Ten or more brains removed from mice showing nervous signs are ground with sand or alundum and 10 cc. of inactivated† undiluted rabbit serum are added for each gm. of brain tissue. The suspension may receive additional homogenization in a Waring blender. After centrifugation at about 2000 r.p.m. for ten minutes, the supernatant fluid is drawn off and regarded as the 1 to 10 dilution of virus. Part of it is titrated immediately, and the rest is distributed in ampules in 1-cc. amounts. The sealed ampules are quickly frozen in a mixture of solid CO_2 and 95 per cent alcohol and stored in a solid CO_2 refrigerator. Depending upon needs, one or more ampules are thawed and used in each test, the unused portion of the virus being discarded.

(b) *Preparation of Virus Dilutions, and Control and Serum-virus Mixtures*.—Starting with the 1 to 10 dilution from the frozen ampule, one prepares 1 to 50, 1 to 500, 1 to 5000, etc., up to 1 to 500,000,000 or 1 to 5,000,000,000 dilutions, using 10 per cent rabbit serum in saline as the diluent and a separate pipet for each dilution. Then 0.2 cc. of the selected dilutions are added to marked tubes containing either 0.2 cc. of the undiluted unknown serum or of a tested negative, undiluted rabbit serum for the control mixtures.

(c) *Selection of Critical Dilutions*.—If the preliminary titration has indicated that the LD_{50} titre of the virus to be used is in the range of the 10^{-8} dilution, after incubation at $37^\circ C.$ for two hours, then the control dilutions to be tested will be 10^{-6} , 10^{-7} , 10^{-8} , and 10^{-9} and the serum dilutions 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} . Similarly:

- (1.) with preliminary LD_{50} titre of virus in range of 10^{-7} ,
 Control dilutions 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8}
 Serum dilutions 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6}

* Reed, L. J., and Muench, H.: *Am. Jour. Hyg.* 27, 493, 1938.

† Inactivation is carried out at $56^\circ C.$ for one-half hour.

- (2) with preliminary LD₅₀ titre of virus in range of 10⁻⁹,
 Control dilutions 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰
 Serum 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸

These dilutions are selected in order to be within the range of 100 per cent mortality at one end, and 100 per cent survival at the other in the control titration, bearing in mind the variations in titre that may be expected with virus suspensions preserved in the frozen state. When the test is to be used for diagnosis with a virus that has been found to maintain its titre well, one may dispense with the lowest dilution in the serum mixtures (*i. e.* the 10⁻⁷, or 10⁻⁸, or 10⁻⁹) and use 3 instead of 4 serum-virus dilutions.

(d) *Incubation, Numbers of Mice, and Period of Observation.*—Five mice are to be inoculated with each dilution. The intracerebral route of inoculation is used, employing 0.25-cc. tuberculin syringes to permit a more accurate delivery of a standard 0.03-cc. dose. If the number of mice should become limited either because of inadequate supply or the large number of sera to be tested, four mice may be used for each dilution. The controls and serum-virus mixtures are to be incubated in a water bath at 37° C. for two hours and then placed in an ice-bath until inoculated. The highest dilutions are to be inoculated first (*i. e.*, 10⁻⁹, 10⁻⁸, 10⁻⁷, etc.) and the control mixtures should be the last. The mice shall be observed and deaths recorded for at least ten days in the case of Western and Eastern equine encephalitis and for at least fourteen days in the case of St. Louis encephalitis virus. All deaths within twenty-four hours after inoculation shall be regarded as being due to traumatic or non-virus causes, and in the case of St. Louis encephalitis virus, considering the dilutions used, all deaths during the first three days shall be regarded as non-specific.

EXAMPLE

Mixture	Final dilution of virus						LD ₅₀ titre, log of dilution	Neutraliza- tion index	Result
	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹			
Control			5/5	4/5	3/5	0/5	8 0		
Serum A	5/5	5/5	5/5	3/5			7 0 +	<10	Negative
Serum B	5/5	5/5	3/5	2/5			6 5	32	Equivocal
Serum C	5/5	4/5	3/5	0/5			6 0	100	Positive

Fraction 3/5 indicates that 5 mice were inoculated and in 3 death occurred as a result of the virus infection.

PROCEDURE USED IN CALCULATING LD₅₀ TITRE*

Control

Accumulation totals

	Died	Survived	Died	Survived	Deaths	% mortality
10 ⁻⁹ - 5,5	5	0	12	0	12/12	100 0
10 ⁻⁸ - 4/5	4	1	7	1	7/8	87 5
10 ⁻⁷ - 3/5	3	2	3	3	3/6	50 0
10 ⁻⁶ - 0/5	0	5	0	8	0/8	0

* LD₅₀ titre, log of dilution = 8.0.

Arrows indicate direction of addition for accumulation totals

* The Reed-Muench formula will be used even in the absence of a zero mortality end-point or of 2 dilutions below the 50 per cent or more mortality range, with the clear understanding that the results are not absolutely accurate. This is the best available procedure for a standard method of expressing the results under circumstances which make it impractical to increase the number of dilutions to cover the strict requirements of the Reed-Muench formula.

Serum B

	Died	Survived	Accumulation totals			
			Died	Survived	Deaths	% mortality
10 ⁻⁴ - 5/5	5	0	15	0	15/15	100 0
10 ⁻⁵ - 5/5	5	0	10	0	10/10	100 0
10 ⁻⁶ - 3/5	3	2	5	2	5/7	71.4
10 ⁻⁷ - 2/5	2	3	2	5	2/7	28 6

$\frac{\% \text{ mortality above } 50\% - 50\%}{\% \text{ mortality above } 50\% - \% \text{ mortality below } 50\%} = \text{factor or proportionate distance}$

or

$$\frac{71.4\% - 50\%}{71.4\% - 28.6\%} = \frac{21.4}{42.8} = 0.5$$

When tenfold dilutions are used LD₅₀ titre = log of dilution above 50% + factor or in this case = 6.0 + 0.5 = 6.5.

Calculation of Neutralization Index for Serum B

Control titre log of dilution = 8 0

minus

Serum B titre log of dilution = 6 5

Neutralization index log = 1 5

Antilog of 1.5 = 31.6 or 32; therefore neutralization index = 32.

III. Complement-fixation Tests.—Analogous to bacterial complement fixation it has been shown that antiviral sera fix complement in the presence of their homologous antigens. Such tests are employed with increasing frequency in many virus diseases.* The difficulty with the virus complement-fixation tests generally concerns the antigen. Infected tissue must often be employed although occasionally antigen can be derived from tissue culture, notably from allantoic fluid, of the developing chicken embryo.

Various tests have been devised for use in infections such as vaccinia and variola; herpes simplex; psittacosis; lymphopathia venereum; influenza; lymphocytic choriomeningitis and various other neurotropic virus infections. For the latter group the method followed by Casals† is recommended. A brief review of this technic is as follows:

The sera to be tested are inactivated at 60° C. for twenty minutes, undiluted or in a dilution 1 to 2.

The antigen is a suspension of infected mouse brain in ten times its weight of saline. This emulsion is kept in the ice-box twenty hours, then it is centrifugalized at 2500 r.p.m. for thirty minutes; the supernatant is frozen and thawed five times in a dry ice-alcohol mixture and then centrifugalized in the angle centrifuge at 3500 r.p.m. for one hour; the supernatant, after the addition of merthiolate in dilution 1 to 1000 is the antigen. These antigens are virulent and precaution should therefore be observed in handling them. In order to avoid certain difficulties and dangers inherent in this (frozen and dried) non-virulent antigens for several can be obtained from one of the biological

* For disease Man. † Cf. Unn

complement-fixation tests in virus
DICK, A. J.: Virus Diseases of

TABLE 80.—PROCEDURES FOR THE ISOLATION OF VIRUS AND FOR SEROLOGICAL DIAGNOSIS IN HUMAN VIRUS INFECTIONS

Case of virus	Type of infection	Insect vector	Natural animal reservoir	Isolation of virus				Diagnostic serological tests in man
				Human specimens to be tested	Animals to be inoculated	Route	Positive result in inoculated animal—Symptoms and pathology	
New disease	Lymphocytic choriomeningitis		House mice ? dogs	Blood Spinal fluid— spinal and brain	Mice Guinea pigs	(Intracerebral Intraperitoneal Intracerebral Subcutaneous Intraperitoneal)	Encephalitis and choroiditis Local secretions of liver and spleen, pneumonia Mild meningitis Death in 9 to 16 days with pneumonia, focal infiltrations in liver	Neutralization Complement fixation
	Rabies		Dogs and many mammals Rats and cattle (Trinidad)	Brain Brain (dog)	Mice Mice	Intracerebral Intracerebral	Encephalitis—Negri bodies present Encephalitis—Negri bodies present	
	Poliomyelitis	? Flies	Man	Stools (Oral washings)	Monkeys	(Intracerebral Intranasal Intraperitoneal)	Experimental poliomyelitis with lesions in mid-brain and (usually) all levels of the spinal cord	
	Japanese encephalomyelitis (Western type)	Mosquito Culicx (and others)	Many mammals and birds	Blood Spinal fluid —Brain	Mice Guinea pigs	(Intracerebral Subcutaneous Intraperitoneal)	(Encephalitis and myelitis	Neutralization Complement fixation
Old disease	(Eastern type)	Mosquito Culicx	Mammals and birds	? Blood —Brain	Mice Guinea pigs	(Intracerebral Subcutaneous Intraperitoneal)	Encephalitis and myelitis	Neutralization Complement fixation
	(African type)	?	Mammals	Oral washings ? Blood and spinal fluid ? —Brain	Mice	(Intracerebral Subcutaneous Intraperitoneal)	Encephalitis	Neutralization
	or Encephalitis	Mosquito Culicx (and others)	Many mammals and birds	Brain	Mice	Intracerebral	Encephalitis	Neutralization Complement fixation

Infection	Inoculation	Host	Site of Inoculation	Inoculum	Reaction	Notes
Ocular	Inclusion blepharitis	Man	Conjunctiva	Monkey	Intraconjunctival	Conjunctival ophthalmia
	Epidemic keratoconjunctivitis	Man	Conjunctiva	Man	Intraocular	Conjunctivitis
	Trachoma	Man	Conjunctiva	Monkey ? Rabbit	Intraconjunctival ? Intraocular	Conjunctival follicles (last 20 to 30 days)
Respiratory	Paratuberculosis (Ornithosis)	Pigeon	Sputum Blood	Pigeon	Intraconjunctival	Death (4 to 11 days), severe lesions with necrosis in lumen, meningococcal-like
	Influenza A	Man	Oral washings	Pigeon	Intraocular	Purulent conjunctivitis
	Influenza B	Man	Oral washings	Pigeon	Intraocular	Purulent conjunctivitis
	Influenza X			Pigeon	Intraocular	Purulent conjunctivitis
	Common cold	Man	Oral washings	Chimpanzee Man	Oral (Oral)	Experimental conjunctivitis
Vasomotor	Mumps	Man	Saliva	Monkey ? Rat		Acute purulent conjunctivitis (experimental mumps)
	Yellow fever	Monkey ? Man	Blood— Liver and spleen	Monkey (Man)	Subconjunctival (Intraocular)	Yellow fever
	Dysentery	Man (? Monkey)	Blood	Monkey Man	Subconjunctival and others	Yellow fever
	Sand-fly fever (? poxvirus)	Man	Blood	Monkey		Yellow fever
	Rift Valley fever	Sheep	Blood	Monkey (Man)	Subconjunctival Intraocular	High mortality with conjunctivitis Transected fever Severe conjunctivitis Lesions with mucous degeneration of liver
	Lymphogranuloma venereum	Man	Lymph gland material	Man	Intraocular	Conjunctivitis

YELLOW FEVER

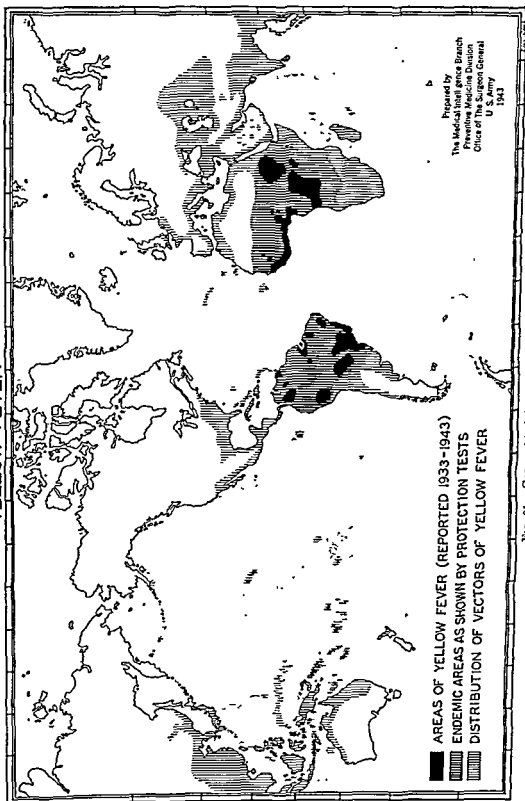


FIG. 01.—Geographical distribution of yellow fever.

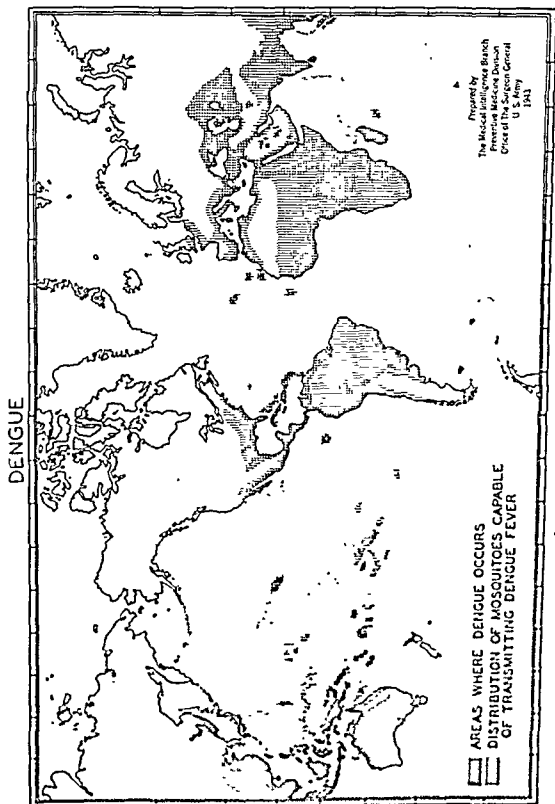


FIG. 62.—Geographical distribution of dengue.

The test is run as follows: the complement is titrated as for the Wassermann reaction: two units are used in the test. Then come the successive steps of the reaction:

(a) Two-fold dilutions, from 1 to 1 or 1 to 2 through 1 to 16, of the inactivated serum are put in as many series of tubes as antigens are available; the volume of the serum dilutions is 0.25 cc.

(b) To each tube in the series 0.25 cc. of a given antigen are added. At least four different antigens can be used each time, namely: W.E.E., E.E.E., L.C.M., and St. Louis encephalitis.

(c) An extra tube is set aside for each antigen, as control of the anti-complementary power of the antigen; this tube contains 0.25 cc. of antigen and 0.25 cc. of saline, the latter to substitute for the serum.

(d) Two units of complement, as determined by the previous titration, are added to *each* tube in a volume of 0.5 cc.

Now the tubes containing dilutions of serum, the antigen, and the complement all in a volume of 1 cc., as well as the tubes containing the antigen and the complement but *no* serum also in a volume of 1 cc. (since 0.25 cc. of saline substitutes for the same volume of serum), are placed in the ice-box for eighteen hours. Following this incubation the tubes are kept at room temperature for one-half hour, and the hemolytic system is then added, consisting of 0.25 cc. of a 3 per cent suspension of packed sheep blood cells and 3 hemolytic doses of anti-sheep hemolysin also in 0.25 cc. The total volume of fluid in each tube is now 1.5 cc. The tubes are incubated in a water bath of 37° C. for one-half hour and the reaction is read.

Another and modified technic for the neurotropic group of viruses is that recommended by Havens, *et al.**

IV. Precipitin Reactions.—These reactions have occasionally been brought out through the use of immune sera prepared from rabbits injected with elementary bodies of vaccinia. In yellow fever, convalescent human sera have been shown to contain a precipitin capable of reacting with precipitinogen occurring in the blood of infected monkeys. The test has not yet found much practical application, however.

* Havens, W. P., Jr., Watson, D. W., Green, R. H., Lavin, G. I., and Smadel, J. E: *Jour. Exper. Med.*, **77**, 139, 1943.

PART VI

Protozoölogy

CHAPTER XXXIV

GENERAL DESCRIPTION OF PROTOZOA

By QUENTIN M. GEIMAN

ORGANISMS belonging to the Phylum Protozoa are of medical importance because numerous species produce disease in both animals and man. Although the greater number of approximately 10,000 species are free living saprophytes, parasitic habitats have been established by representatives of each class of Protozoa. Species may live as ectoparasites or as endoparasites in harmless or disease-producing association with their hosts.

Before proceeding with a consideration of pathogenic species, the diseases they produce, and methods of diagnosis, a brief description of Protozoa as animals is desirable. Since the organisms in this phylum vary in their size, morphology, organization, and physiology, a brief classification will be tabulated to serve as a guide for the laboratory worker.

I. Morphology.—Protozoa are unicellular animals composed of nucleus and cytoplasm. Difficulties arise, however, when an attempt is made to distinguish some species from unicellular plants and certain Metazoa. As might be expected, gradations of differentiation exist in the evolutionary scale of Protozoa and borderline forms are not always classified easily according to our arbitrary system of nomenclature. Protozoa vary from simple organized cells with nucleus and cytoplasm to colonial aggregates with complex organization.

In the evolutionary development of the Protozoa, a variety of specialized structures or organelles have developed. In organisms such as the amœbæ,

sions making individual organisms visible to the naked eye. Amœbæ constantly change shape in the active stage but other species maintain more or less constant form for the many and diverse stages which go to make up complex life-cycles. Adaptation to mode of existence, both morphologically and physiologically, is a prime requisite for the successful parasite.

1. The Cytoplasm.—The cytoplasm is a viscous fluid which contains the inclusions and organelles and which makes up the greatest volume of the cell. This fluid has the properties of a complex colloidal system composed of organic and inorganic constituents. Layers of different refractivity and viscosity may be distinguished as ectoplasm and endoplasm. A plasma

membrane bounds the fluid in some cases and a periplast or cuticle in other cases.

The structural components differ in origin, nature, and function. The presence of various types of granules, crystals, pigment, mitochondria, fibrils, stored food, and vacuoles varies from species to species. Particulate food or nutrient substances that are ingested or absorbed by osmosis give rise to the waste products of metabolism. These waste products may be thrown off by a contractile vacuole or excreted directly through the cell membrane. The metabolism of parasitic Protozoa is of considerable importance to the host, because the pathogenicity of the parasite may be directly correlated with the utilization of nutrients from the environment and the toxicity of excretory and secretory products.

Direct observation of the cytoplasm provides limited information about the cell constituents. Special staining procedures are required to differentiate the structures present. Physiological and biochemical methods are needed to provide more complete knowledge about the processes which take place in this complex fluid.

2. **The Nucleus.**—The nuclei of Protozoa vary in size and structure from species to species. This essential component of the protozoan cell contains karyolymph or nuclear sap, a reticulum or network, chromatin dispersed in different ways and a nuclear membrane. In some species of flagellates, accessory chromatic structures are associated with the nucleus and the flagella or organs of locomotion. In the more highly differentiated ciliates, a large macronucleus and a smaller micronucleus, each with specialized functions in cell division and reorganization, comprise the nucleus. Further generalizations are difficult to make because of the diverse nuclear structures and organization in relation to the cytoplasm. Textbooks of protozoology will have to be consulted for more complete information.

II. Reproduction.—Reproduction, as a life process, is also varied and complex. Although generalizations about it are difficult to make, knowledge of the process is of fundamental importance to an understanding of the parasitic Protozoa and their pathogenicity.

The main types of division are asexual and sexual, with an additional type involving the reorganization of ciliates by the temporary fusion or conjugation of two individuals.

Asexual multiplication by binary fission, budding, and multiple division or segmentation is quite common among the Protozoa. Nuclear division precedes the division of the organism into two equal or unequal daughter cells, or segmentation into many cells. The latter process, called schizogony, occurs mainly among the Sporozoa, of which the parasite of malaria is an example.

Sexual division involves a differentiation of sexes or gametes and the union or copulation of two cells to form a zygote. This zygote can proceed to encyst or undergo a process of sporogony (development of spores). The temporary fusion of two individuals, known as conjugation, permits nuclear exchanges which give rise to the reorganization of the involved cells and increased vitality for subsequent binary fission. This latter process is characteristic of the ciliates only.

The above processes can be involved in the life-cycle of many Protozoa. The occurrence of sexual and asexual methods of reproduction in the same species is known as alternation of generations. This process is of

particular importance to the parasitologist because of the change of host frequently associated with it. The new host is usually an arthropod which becomes the transmitter or vector for transfer of infection from one individual to another. Drastic changes of environment involved in the transfer from vertebrate to invertebrate host requires prompt adaptation, if the protozoan is to survive. That such infections continue to exist indicates the readiness with which the parasite adapts itself to the respective hosts involved in the life-cycle.

III. **Classification.**—Although present-day classification of the Protozoa differs slightly from author to author, the organisms, with the exception of the class Sporozoa, may be classified by their method of locomotion. The table listed below includes only those groups of organisms which are of importance as parasites of man.

TABLE 81.—CLASSIFICATION OF THE PROTOZOA*

Phylum: Protozoa

A. **Subphylum:** *Plasmodroma*, Doflein, 1901. Movement is effected by pseudopodia or flagella, and syngamy, where it is known, takes place by the complete

dominating phase is amoeboid,

dominating phase is flagellate,

ic forms which are frequently provided with polar capsules

IV. **CLASS:** *Sporozoa*, Leuckart, 1879. Parasitic forms which reproduce typically by schizogony, and which give rise to sporozoites enclosed in resistant oöcysts after syngamy has occurred.

B. **Subphylum:** *Ciliophora*, Doflein, 1901. Movement is effected by means of cilia.

Group 1:

all of one type

I. **CLASS:**

Group 2:

which develop into temporarily associated individuals.

I. **CLASS:** *Ciliata*, Perty, 1852. Cilia are present throughout the life of the organism.

II. **CLASS:** *Suctorii*, Claparede and Lachmann, 1858. Cilia are present only during the young stages, which usually attach themselves to objects, lose their cilia, and develop suctorial tentacles.

* WENTON, C. M.: *Protozoology*, London, Baillière, Tindall & Cox, 1926

CHAPTER XXXV

INTESTINAL PROTOZOA

By QUENTIN M. GEIMAN

GENERAL

BEFORE proceeding to methods for diagnosis of human intestinal Protozoa, some knowledge of the biology of these organisms is required. The general morphology has been considered briefly, but physiology is of considerable importance too because of the necessity for examining freshly collected specimens, cultivation for isolation of pathogens, study of experimental infections, and transmission and control. Throughout these procedures, the specimens or cultures of the parasites must be handled as living cells derived from a host with a body temperature of 37° C. Diluents and culture media should be isotonic and hydrogen ion concentration should be properly controlled.

Procedures for
logical methods.
sterile technics should be followed throughout. Proper handling of the microscope is essential because the organisms are minute and their morphological characters must be studied for identification. A calibrated ocular micrometer is essential for the microscopist to aid in establishing size relationships and to escape the many pitfalls which occur as artefacts in specimens requiring microscopic examination. Precise methods of technic and observation are the forerunners of reliable and accurate diagnosis.

SPECIES OF PROTOZOA

Those species of Protozoa to be found in the intestinal tract of man are listed in Table S2. Three exceptions are included, *Endamaba gingivalis* and *Trichomonas tenax* which occur in the mouth only, and *T. vaginalis* which occurs only in the vagina of women and the urethra of men. Even though only three species, *Endamaba histolytica*, *Trichomonas vaginalis* and *Balantidium coli*, are of proved pathogenicity, knowledge of the other species is necessary in order to differentiate the non-pathogenic from the pathogenic. First-hand experience with the plant-like organism *Blastocystis hominis* (Fig. 65, Nos. 10 and 11) and with the variety of appearances presented by fecal debris is also essential for differentiation.

I. Description of Parasites.—1. *Endamæba histolytica* (Schaudinn, 1903) Hickson, 1909.—This organism has three stages in its life cycle, the active or trophozoite stage, the precystic stage and the cystic stage. The trophozoites are the tissue invaders and the cause of amœbic dysentery. The cysts are the resistant and infective stages which lead to infection of new hosts. Two races, a large (Fig. 63, Nos. 1 to 6) and a small (Fig. 63, Nos. 7 to 11) race, occur, but the large race is more commonly found.

(a) *Trophozoite*.—Size, 15 to 60 μ in diameter, with an average of 20 to 30 μ . In fresh warm feces, the organisms are rapidly motile with a snail-

like movement, thrusting out clear ectoplasmic pseudopodia upon change of direction. The ectoplasm may or may not be differentiated from the less refractive endoplasm and the nucleus is seldom visible. The organisms ingest food particles by phagocytosis and red blood corpuscles may be taken in if they are present in the stool. Vacuoles and bacteria are visible in sluggish, degenerating organisms.

TABLE 82.—CLASSIFICATION OF INTESTINAL PROTOZOA

Class	Order	Phylum Protozoa	
		Genus	Species
Rhizopoda	Amœbida	<i>Endamaba</i> —Leidy, 1879	<i>E. histolytica</i> (Schaudinn, 1903), Hickson, 1909
			<i>E. coli</i> (Grassi, 1879), Hickson, 1909
			<i>E. gingivalis</i> (Gros, 1849), Smith and Barrett, 1915
		<i>Iodamaba</i> —Dobell, 1919	<i>I. butschii</i> (v. Prowazek, 1911), Dobell, 1919
		<i>Endolimax</i> —Kuenen and Saeffengrebel, 1917	<i>E. nana</i> (Wenyon and O'Connor, 1917), Brug, 1918
Mastigophora	Protomonadida	<i>Dientamaba</i> —Jepps and Dobell, 1918	<i>D. fragilis</i> Jepps and Dobell, 1918
		<i>Giardia</i> —Kunstler, 1882	<i>G. lamblia</i> (Stiles, 1915)
		<i>Chlamastix</i> —Alexeieff, 1910	<i>C. mesnii</i> (Wenyon, 1910), Alexeieff, 1912
		<i>Trichomonas</i> —Donné, 1836	<i>T. hominis</i> (Davaigne, 1860), Leuckart, 1879
		<i>Retortamonas</i>	<i>T. tenax</i> (O. F. Müller, 1773)
Ciliata	Heterotrichida	<i>Enteromonas</i> —da Fonseca, 1915	<i>E. intestinalis</i> , Wenyon and O'Connor, 1917
		<i>Balanitidium</i> —Claparède and Lachmann, 1858	<i>E. humanis</i> , da Fonseca, 1915
Sporozoa		<i>Isospora</i> —Aime Schneider, 1881	<i>B. coli</i> (Malmsten, 1857), Stein, 1862
			<i>I. hominis</i> (Rivolta, 1878), Dobell, 1919

In stained preparations, the distinctive morphology characteristic of the species becomes apparent (Fig. 63, Nos. 1 and 2). The addition of iodine to a fresh preparation, stains the nucleus so that the central karyosome and the fine peripheral chromatin can be detected for diagnostic purposes. Permanent preparations stained with Heidenhain's iron-alum hematoxylin give the most precise definition of the nuclear morphological detail (Fig. 63, No. 1). The small central karyosome or dot of chromatin and the fine peripheral chromatin just beneath the nuclear membrane are diagnostic for the species. If preparations are made from fecal specimens more than one hour old, degeneration of the nucleus takes place with the chroma-

The size of cytoplasm

Highly refractile and deeply staining cylindrical rods or chromatoid bodies and a large glycogen vacuole, staining brown with iodine, are formed in the cytoplasm.

(b) *Cyst*.—Size, 5 to 20 μ in diameter. Averages of large race 13.5 μ , small race 7 to 9 μ . Cysts are spherical containing 1, 2 or 4 nuclei, chromatoid bodies, and glycogen vacuole (Fig. 63, Nos. 3 to 6). The single nucleus divides in two and then each nucleus divides again to form four nuclei. During this division process, the chromatoid bodies and the vacuole decrease in size and eventually disappear. The mature cyst, which can produce infection when ingested, contains clear cytoplasm and four nuclei (Fig. 63, No. 6). The number of nuclei and characteristic chromatoid bodies are diagnostic.

(c) *Life History*.—Cysts passed in feces by individuals with chronic dysentery or by carriers remain viable, if kept moist, for varying lengths of time up to several weeks. These cysts, when ingested with contaminated food and water by a new host, pass through the stomach and excyst in the small intestine. A metacystic amœba containing the four cystic nuclei

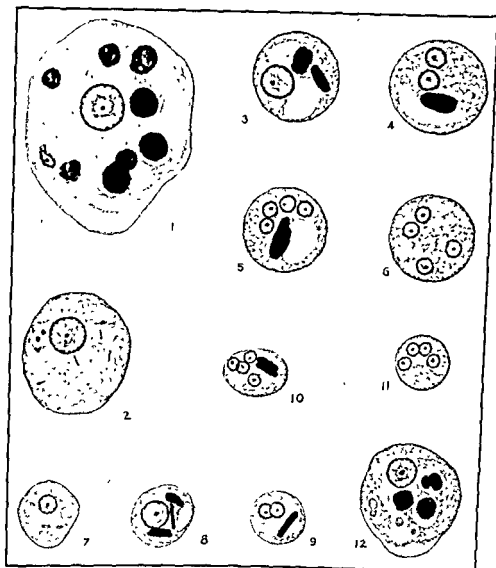
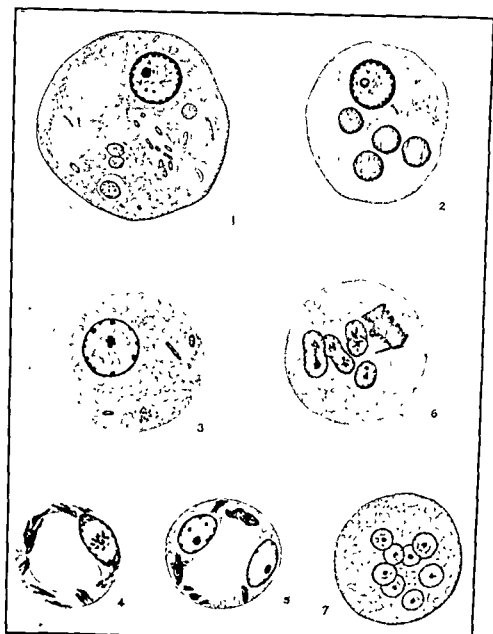


FIG 63—Various stages of *Endamaba histolytica* ($\times 2000$). 1, Trophozoite containing red blood corpuscles, 2, precystic stage, 3, cyst containing one nucleus, chromatoid bodies and a glycogen vacuole, 4-6, typical binucleate and quadrinucleate cysts; 7, trophozoite (small race) of *E. histolytica*; 8-11, cysts (small race) of *E. histolytica*, 12, trophozoite of *E. gingivalis*. (Heidenhain's iron-alum hematoxylin stain) (Original)

emerges from each cyst. A somewhat complex metacystic development results in the ultimate production of eight small amœbulæ from each metacystic amœba. These amœbulæ grow and divide by binary fission to produce typical active trophozoites which can initiate tissue invasion and production of disease once again in the large intestine. Whenever required

conditions for cyst formation develop, cysts are formed and passed in the feces to repeat the life cycle.



The incidence of *E. histolytica* among the general population depends to some extent on the local sanitation. In the United States, infection rates vary from 2 to 10 per cent or higher, but percentages approach 25 per cent or higher in tropical areas.